CLINICAL CHEMISTRY

Journal of the American Association of Clinical Chemists

VOLUME 3

1957

PAUL B. HOEBER, INC., PUBLISHERS

MEDICAL BOOK DEPARTMENT OF HARPER & BROTHERS

CLINICAL CHEMISTRY

Journal of the American Association of Clinical Chemists

 For information address PAUL B. HOEBER, INC., Medical Book Department of Harper & Brothers, 49 East 33rd Street, New York 16, N. Y. Printed in the United States of America

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CLINICAL CHEMISTRY

Journal of the American Association of Clinical Chemists

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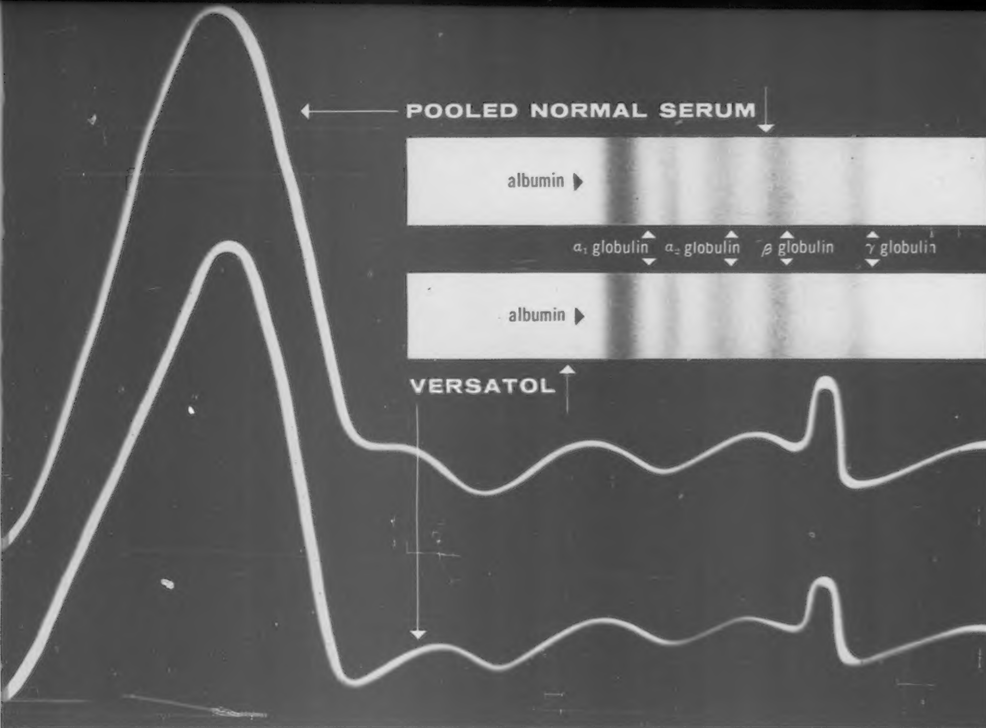

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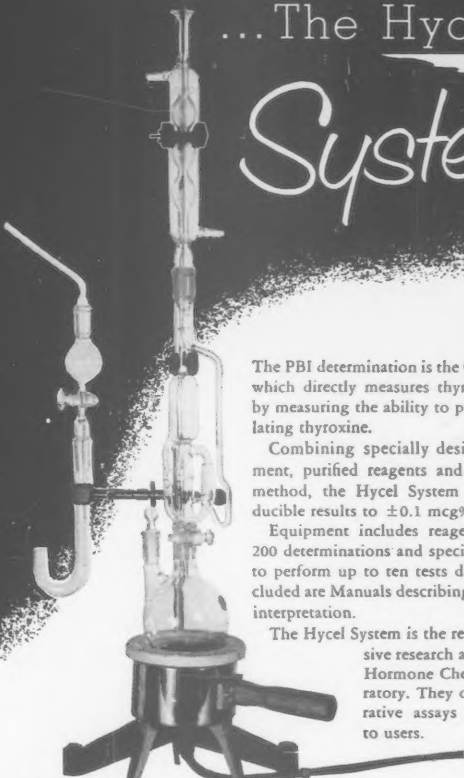

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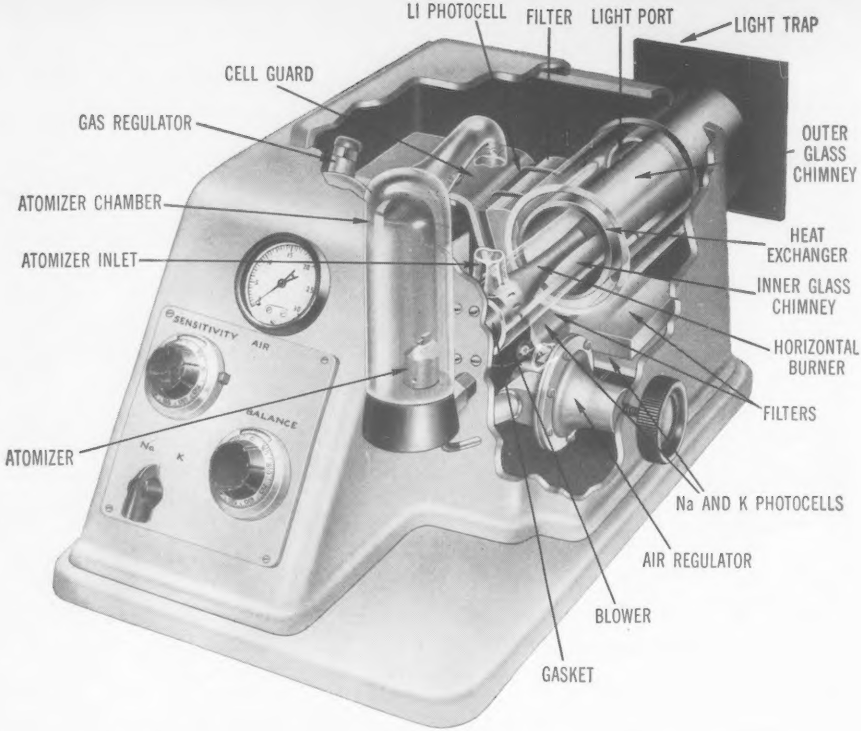
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Identification of Human Hemoglobins

C. A. J. Goldberg

DIFFERENCES IN THE HEMOGLOBINS of the adult and the newborn have been recognized since 1866 (36). Rapid advances in this field, however, began in 1949, when Pauling and Itano (25) discovered that the hemoglobin of sickle cell disease differs from adult hemoglobin. Since that time, 8 hemoglobins have been identified, hemoglobin C (18) in 1950, hemoglobin D (14) in 1951, hemoglobin E (7, 17) and hemoglobin G (10) in 1954, and hemoglobin H (24, 28) in 1955. Hemoglobin I was described by Rucknagel and coworkers (30) in 1955, hemoglobin J by Battle and Lewis (2) in 1954. The hemoglobin listed as K by Allison (1) carries the designation J in the review of Itano (16), and is further described by Thorup (1, 16).

The heterogeneity of normal adult hemoglobin as manifested by its electrophoretic behavior was first described by Derrien (9) in 1953. Studies by Kunkel and Wallenius (19), and Shavit and Brener (31) supported this finding. Morrison and Cook (22) observed the heterogeneity of hemoglobin A by chromatography on an ion-exchange resin. More recently Cook and Morrison (8) have found hemoglobin F to be heterogeneous as well.

The alkali-resistant hemoglobin present in normal adults in very small amounts appears to be identical with fetal hemoglobin in amino acid composition according to studies made by Huisman *et al.* (12). Van der Schaaf and Huisman (34) have found that the alkali-resistant component present in some patients with sickle cell anemia differs from fetal hemoglobin in ultraviolet absorption spectra and amino acid composition.

Excellent reviews on the present status of the human hemoglobins by Chernoff (4, 5, 6) and Itano (16) have appeared in 1955.

From the William Pepper Laboratory of Clinical Medicine, University of Pennsylvania, Philadelphia, Pa.

Received for publication April 24, 1956.

Although electrophoresis at pH 8.6 is the most important single method for the identification of the various hemoglobins, a number of supplementary methods are required. The following methods will be discussed in this paper: electrophoretic separations at various H-ion concentrations, together with a method for quantitative measurement of the hemoglobins, chromatography, measurement of alkali denaturation, determination of the solubility of reduced hemoglobin, and sickling tendency.

ELECTROPHORESIS

Two methods for electrophoretic separation of hemoglobins will be described. Method I, in which an improved pressure-plate apparatus is used, is the method of choice. Method II is for use with apparatus in which the paper strips are suspended horizontally. Difficulties in separating hemoglobins by the conventional procedures in the latter have caused many workers to turn away from this method. However, it can be adapted for hemoglobin separations according to the technic described below.

Factors Involved in Electrophoretic Separation of Hemoglobins

The factors which influence the separation of hemoglobins are many. Those of particular importance may be listed in an equation as follows:

$$d_a - d_b = F \frac{tRE}{Dr/2} \qquad D = \frac{I}{q}$$

where $d_a - d_b$ = displacement of 2 different hemoglobins
t = time
R = resistance
E = field strength (potential gradient)
q = width of the paper in cm.
I = current in amperes
D = current density
$r/2$ = ionic strength of the buffer system
F = factor (to include all other variables which are not of primary importance as *f.i.* temperature)

Time and Field Strength. The degree of separation of the hemoglobins is directly proportional to time and field strength. The optimal field strength for the methods described was found to be 4–6.5 v./cm. Experiments carried out over a time period of 15–20 hours at this field

strength will provide better patterns than experiments at higher potentials for a shorter time. However, the time should not exceed 20 hours, to prevent excess diffusion.

FRICTIONAL RESISTANCE. The resistance to the flow of buffer through the paper should be high. This may explain why hemoglobin electrophoresis on compressed paper or on paper suspended over a ridge pole has been so much more satisfactory than on freely suspended horizontal paper strips, for buffer flow in the former is much less than in the latter. It is obvious therefore that the frictional resistance should be increased to obtain the satisfactory separation of hemoglobins on a free-hanging horizontal strip. This may be done in several ways. Paper may be compressed by means of a glass rod attached to the bridge by a strong rubber band (Fig. 1), or the paper strip may be cut to decrease the width of the paper hanging in the buffer solution (Fig. 2). A favorable buffer flow is obtained in the latter when the paper is cut down to one fourth of its original width. The frictional resistance may be increased further by the addition of nonelectrolytic solutes to the buffer system to increase the viscosity of the buffer. The effect of three substances was investigated: sucrose, dextran, and glycerol. As may be seen from Table 1, the effect of the addition of 3% dextran on the viscosity of the Veronal buffer slightly exceeds that of 25% sucrose. The effect of 5% glycerol is slightly less than that of 10% sucrose.

The effect of these substances on the migration of the hemoglobins is a decrease in the total distance of migration with a negligible loss of resolution in pressure-plate types of apparatus. This effect is very beneficial when working at higher temperatures. The total distance of migration and the resolution of hemoglobins A, S, and C at 28° when 25% sucrose is added to the Veronal buffer is about the same as at 10° when Veronal buffer only is used (see Table 1). It is possible therefore to obtain satisfactory and comparable patterns of various hemoglobins at different temperatures by adding sucrose in amounts varying with temperature. Keeping the potential constant at 10° Veronal buffer may be used without any additional solute. At 20°, 10% sucrose may be added, and for temperatures around 30° the addition of 25% sucrose will result in satisfactory patterns.

Dextran proved to be less suitable in that it caused greater diffusion in the hemoglobin patterns. Little benefit was derived from the addition of dextran in amounts smaller than 3%. On the other hand, when 6% dextran was added to the buffer at a temperature of 28°, there was a complete loss of resolution. The effect of dextran cannot be ascribed to

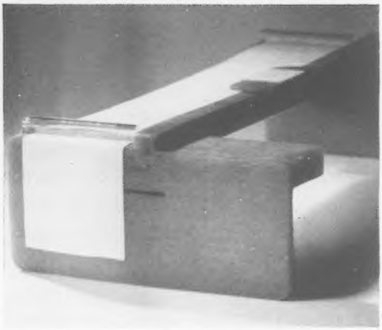
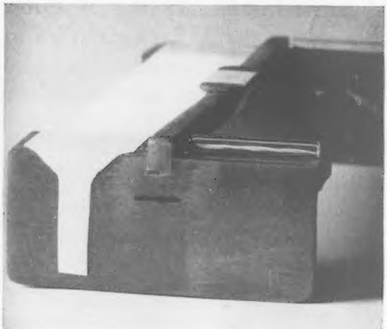
1 
 2

Fig. 1. Paper attached to bridge. **Fig. 2.** Paper cut to decrease the width of hanging end

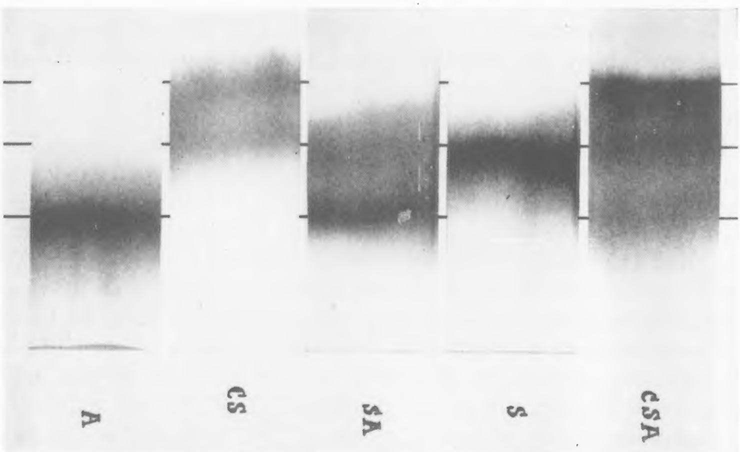
5 

Fig. 5. Separation of hemoglobins on freely suspended horizontal strips.

Plate 1

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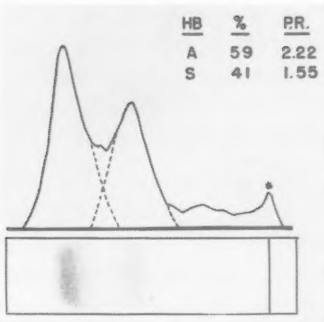

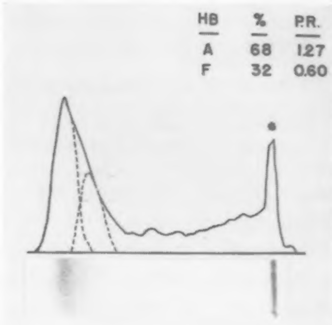

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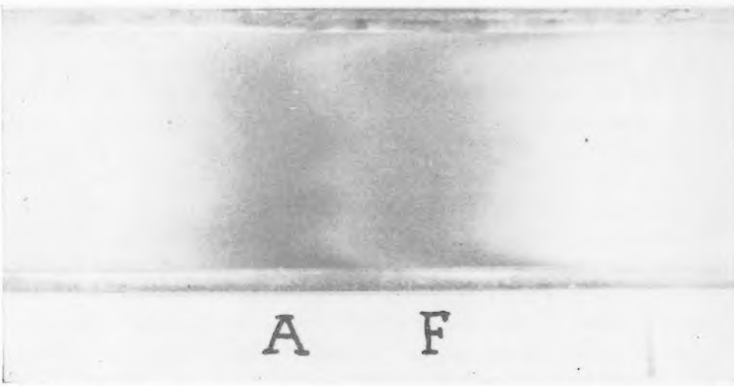

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Fig. 6. Separation of hemoglobins on paper strips enclosed between pressure plates. **Fig. 7.** Galvanometer readings plotted against distance, yielding gaussian curves. **Fig. 8.** Gaussian curve showing distortion of a major peak. **Fig. 10.** Fetal hemoglobin shown by chromatography. Two completely separated zones.

Plate 2

Table 1. Displacement of Various Hemoglobins at 10° and 28° in Different Media, at 250 v., 16 Hr.

Buffer	Solute added	Temp.	Relative viscosity[a]	Hemoglobin A (Displacement in mm.[b])	S	C
Veronal	..	28°	1.00	107	83	54
Veronal	10% sucrose	28°	1.18	98	73	45
Veronal	25% sucrose	28°	1.82	62	47	32
Veronal	6% dextran	28°	3.20	13	..	..
Veronal	..	10°	1.00	70	51	29
Veronal	10% sucrose	10°	1.18	60	38	20
Veronal	25% sucrose	10°	1.82	32	24	13
Veronal	1½% dextran	10°		66	46	32
Veronal	3% dextran	10°	2.00	58	44	30
Veronal	5% glycerol	..	1.11	..	..	..

[a] Measurements made in Ostwald viscosimeter.

[b] Experiments performed with the EC305 of the E-C Apparatus Company.

mere viscosity. When paper strips containing dextran buffer were stained with protein dyes, they retained a great deal more dye than those containing Veronal or sucrose-Veronal buffer. Hence, it may be assumed that there is interaction between the dextran and the paper, and perhaps between the hemoglobins and dextran as well.

When freely suspended horizontal strips were used for the separation of hemoglobins, the effect of the addition of sucrose to the Veronal buffer was not as satisfactory as it proved to be for the pressure-plate apparatus. Sucrose in concentrations of 25% or dextran in concentrations of 3% appeared to have a "drying" effect on the paper resulting, when dextran was used, in charring of the paper in the center, at the sample application site.

Glycerol was substituted in an effort to control evaporation (21, 27) while increasing the viscosity. It was used in amounts varying from 1 to 10 volumes per 100 volumes of Veronal buffer. The sharpest patterns, with the best resolution and least diffusion, were obtained with 5 volumes of glycerol per 100 volumes of Veronal buffer.

Current Density. The rate of separation of the various hemoglobins is inversely proportional to the current density. Hence, the current density should be kept as low as possible. It should not exceed 0.5 ma. per cm. of strip width. For hemoglobin separations on free-hanging horizontal strips, Whatman paper No. 1 is to be preferred to Whatman paper 3MM since it gives a current density half that obtained with the thicker paper at the same potential. Excessive current density will

manifest itself by a rapid rate of migration of the hemoglobins without any separation.

IONIC STRENGTH AND BUFFER SYSTEMS AT *p*H 8.6. The following buffers were investigated: (1) boric acid–potassium hydroxide, *p*H 8.6, 0.025M; (2) borate-phosphate buffer, *p*H 8.6, 0.017M; (3) borate-chloride-NaOH buffer, *p*H 8.7, 0.058M; (4) Michaelis buffer [as described for protein electrophoresis (35)] diluted 1:2 with distilled water *p*H 8.6, 0.05M; (5) barbital buffer (see under methods), *p*H 8.6, 0.06M.

Although separations may be obtained with the borate-phosphate buffer and the dilute Michaelis buffer, the barbital buffer is to be preferred since it causes less trailing and diffusion.

Since the rate of separation of the hemoglobins is inversely related to the ionic strength of the buffer, low ionic strength will be preferable. However, with lowered ionic strength, diffusion becomes an increasing problem. The optimal ionic strength should be determined for each buffer system. For the barbital buffer of *p*H 8.6, it is 0.06.

*p*H. Difficulties in separating hemoglobins by electrophoresis arise mainly from the fact that the isoelectric points of most hemoglobins differ only slightly, with the exception of that of hemoglobin H (Table 2).

Electrophoresis of the hemoglobins at *p*H 8.6 permits no distinction to be made between hemoglobins S and D, F and G, A and J, and I and H (Fig. 3). It has been recommended by Battle and Lewis (2) that electrophoresis be carried out also in a phosphate buffer at *p*H 7.8, which will result in separation of hemoglobins A and J (see Fig. 3). Furthermore, a third electrophoretic pattern carried out in a phosphate

Table 2. SOME CHARACTERISTICS OF THE VARIOUS HEMOGLOBINS

Hemoglobin	I.E.P.[a]	Alk. Res.[b]	F.S.H.[c]
A	6.87	..	High
S (B)	7.09	..	Low
C	7.30	..	High
D	..	..	High
E	..	..	High
F	6.98	Yes	High
G	6.98	..	?
H	5.6	..	Low
I	..	..	High
J	..	Moderate	?
K	..	?	?

[a] Isoelectric points obtained from the literature.
[b] Alkali resistance as determined by the method of Singer (see text).
[c] Ferrohemoglobin solubility, modified Itano method (see text).

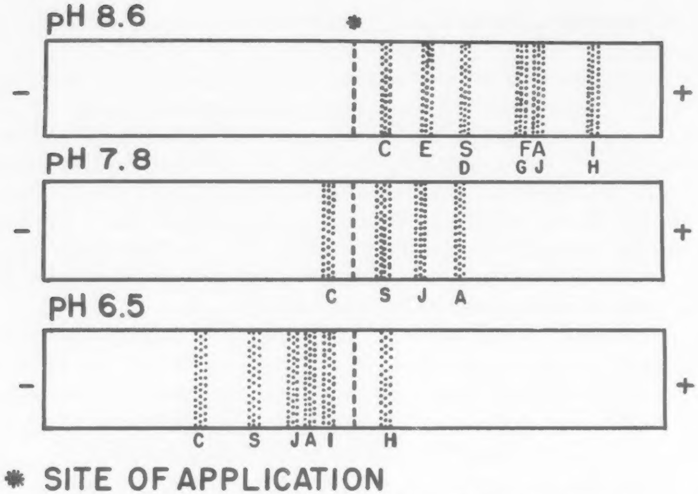

Fig. 3. Electromigration of hemoglobins. Schematic diagram.

buffer at pH 6.5 as suggested by Rucknagel *et al.* (30), will separate hemoglobins I and H, H being the only one to migrate anodically at this pH.

Preparation of the Hemoglobin Solution

Place 4 or 5 ml. of oxalated blood into each of two graduated centrifuge tubes of 15-ml. capacity and centrifugalize for 10 minutes. Withdraw the supernatant and add 10 ml. of saline to the packed cells. Stir gently and again centrifuge for 10 minutes. Withdraw the supernatant and wash the cells three more times with 10-ml. portions of saline (33). To a small portion of washed packed cells add 2 volumes of distilled water, and stir gently until hemolysis is complete. Centrifuge for 10 minutes. Use the clear hemoglobin solution for electrophoresis without delay. To the remainder of the washed packed cells in the graduated centrifuge tubes, add 2 volumes of barbital buffer pH 8.6, stir gently, and transfer the suspensions to two labeled freezer-storage tubes. Keep the samples frozen until needed (the minimal time is overnight). Hemolysis is effected by thawing. Thaw one tube either in the refrigerator or at room temperature. The tube may be warmed to body temperature, but should be cooled at once as soon as all ice has disappeared. *Do not put the tube in warm or hot water!* The sample is centrifuged for 10 minutes and the clear hemolysate is used for further testing.

Hemoglobin samples are apt to deteriorate rapidly; they should be kept in a refrigerator. The aqueous hemolysate deteriorates more rapidly than that buffered at *p*H 8.6. It should be used for electrophoresis on the day it is prepared. If electrophoresis cannot be done at once or is to be repeated, buffered hemolysate should be used. However, if hemoglobin H is present it will not be detected in the buffered hemolysate since it is destroyed by freezing (29).

Frozen samples of hemoglobin will keep for several months, however, refrigerated samples may deteriorate after one week.

Method I. Electrophoresis Using a Pressure-Plate Apparatus

Apparatus

The EC305 apparatus, manufactured by the E-C Apparatus Company, 23 Haven Avenue, New York, was used. It consists of two Plexiglas pressure plates arranged so that cooling water can circulate through the plates in close proximity to the filter paper strips. The buffer compartments are divided into three independent sections. This permits simultaneous separations in different buffer systems to be made if desired. A foam rubber pad between the top and bottom plates distributes the pressure uniformly and compensates for unevenness of the pressure plates or the paper. Local variations in buffer saturation of the paper are eliminated in this way. The electrodes consist of platinum strips separated from the paper by two baffles. Pressure is applied by means of an adjustable clamp attached to the center of the pressure plates. The power supply recommended is that supplied by the E-C Apparatus Company for use with the EC305 electrophoresis chamber.

Reagents

BARBITAL BUFFER *p*H 8.6. Dissolve 20.6 Gm. of sodium barbital and 2.8 Gm. of barbital (diethylbarbituric acid) in carbon dioxide–free distilled water. Adjust the volume to 2 l. This buffer has an ionic strength of 0.06M.

BARBITAL-SUCROSE BUFFER. Add 500 Gm. of sucrose (or less as required) to the preceding before adjusting the volume to 2 l. (see text).

PHOSPHATE BUFFER *p*H 7.8. Dissolve 0.587 Gm. monobasic sodium phosphate ($NaH_2PO_4 \cdot H_2O$) and 6.50 Gm. of dibasic sodium phosphate (Na_2HPO_4) *or* 16.38 Gm. of dibasic sodium phosphate ($Na_2HPO_4 \cdot 12H_2O$) in carbon dioxide–free distilled water. Adjust the volume to 2 l. The ionic strength of this buffer is 0.12M.

PHOSPHATE BUFFER *p*H 6.5. Dissolve 3.11 Gm. of monobasic potassium

phosphate (KH_2PO_4) and 1.49 Gm. of dibasic sodium phosphate (Na_2HPO_4) *or* 3.76 Gm. of dibasic sodium phosphate ($Na_2HPO_4 \cdot 12H_2O$) in carbon dioxide–free distilled water. Adjust the volume to 1 l. The ionic strength of this buffer is 0.1M.

PHOSPHATE-SUCROSE BUFFERS. Add 250 Gm. (or less as required) of sucrose to the preceding before adjusting the volume to 1 l. (see text).

SODIUM CHLORIDE SOLUTION. 0.9 Gm. per 100 ml. This solution is referred to as "saline" in the text.

HEMOGLOBIN REFERENCE SOLUTIONS. Solutions of hemoglobins A, S, and/or C.

Preparing the Chamber

Buffer solutions are poured in the three buffer compartments on one side of the apparatus. With the stopcocks on the leveling tubes open, some of the buffer is allowed to flow to the opposite side of the apparatus, to make sure that there is no airseal in any of the connecting tubes. The compartments on the other side of the instrument are now filled with buffer and the level is adjusted on both sides to about ½ inch over the lower baffle. The buffer levels are allowed to equilibrate for about 1 hour.

Procedure

Strips of filter paper Whatman 3 MM, 18½ x 2½ inches, are dipped in buffer, blotted between layers of filter paper, and placed on a teflon sheet on top of the foam rubber pad in the chamber. Rest the ends of the paper on the end walls of the apparatus and do not immerse them in buffer at this stage. Apply about 10 μl. of hemoglobin solution to the center of the paper in a straight line, working swiftly. A mechanical applicator such as the EC412 (E-C Apparatus Company) or the Spinco applicator is very useful. Another, though less satisfactory, way is to apply the samples as spots. Several samples may be applied to a single strip of paper. One or more controls consisting of known hemoglobins should be included with each run. When all samples are applied, the ends of the paper are bent downward and immersed in the buffer solution. The strips are covered with a Teflon sheet. The lid is closed and the clamping device applied to yield maximum pressure. The clamps on the leveling tubes are closed and the circulation of tap water is started through the top and bottom plates. A current of 250 v. is applied (corresponding to about 6.3 v./cm. potential gradient) for 17 hours.

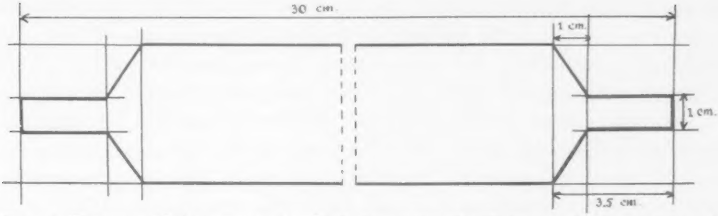

Fig. 4. Diagram of the manner in which the paper should be cut for hemoglobin electrophoresis on a freely suspended horizontal strip.

At the end of the experiment, the current is disconnected, and the lid is tilted carefully to drain off condensate which must not be allowed to come in contact with the paper strips. The Teflon sheet covering the paper strips is lifted off and the ends of the papers are trimmed to the size of the Teflon sheet on which they rest. The strips are allowed to dry in situ on the bottom Teflon sheet. *Do not heat the strips.*

Method II. Electrophoresis Using a Freely Suspended Horizontal Strip

Apparatus

Two types of apparatus were used; that described by Grassmann and Hannig (Bender and Hobein, Munich, Germany) and the apparatus manufactured by the A. H. Thomas Company (Philadelphia, Pa.). Strips of filter paper Whatman No. 1, $1\frac{1}{2}$ x 16 inches, are used for either of these. The ends of the paper may be cut as described in Fig. 4, or the uncut paper may be compressed as shown in Fig. 2.

Reagents

BARBITAL-GLYCEROL BUFFER. Add 50 ml. of glycerol to 1 l. of the barbital buffer described under method I.

PHOSPHATE-GLYCEROL BUFFERS. Add 50 ml. of glycerol to 1 l. of the phosphate buffers described in method I.

Preparing the Chamber

Buffer is poured into the electrophoresis chamber with the bridge in place. Buffer levels are adjusted by means of a leveling tube, as described for the electrophoresis of serum proteins (35).

Procedure

Paper strips, cut as preferred, are dipped in glycerol buffer, blotted, and applied to the bridge. For compressed-paper strips, the glass rod is

lifted slightly and the damp paper is slipped underneath it. *Before* releasing the glass rod, the paper is gently pulled straight and taut. Care should be taken that the paper strip is compressed over its *entire* width, on both sides of the bridge. The bridge is placed in the chamber and the lid is closed. A direct current of 150 v. is applied, resulting in a potential gradient of about 6.3, when the A. H. Thomas apparatus is used. When the Grassmann apparatus is used, the voltage-regulator knob should be turned to its maximum (the maximum output of the Grassmann power supply is 115–120 v.). The stopcock on the leveling tube is left open. After 1 hour the hemoglobin samples are applied to the paper strips, at the center, by means of small applicator strips of 2 mm. width as described previously (35). The stopcock on the leveling tube is closed and the hemoglobins are permitted to migrate overnight. The optimal temperature for hemoglobin separations is 10–20°. At temperatures over 30° and below 5° separations are poor. At the end of the migration period, the current is disconnected and the bridge is lifted out of the chamber. The ends of the paper are gently blotted against the bridge and the patterns are allowed to dry at *room temperature.*

Interpretation of the Patterns

The hemoglobin zones are clearly visible on the paper strips. They may be tentatively identified by inspection and comparison with the reference hemoglobins (Figs. 5 and 6). To measure the amounts of each, the strips may be passed through a densitometer (Photovolt or its equivalent) at 2-mm. intervals, using a 420-mμ filter. By plotting the galvanometer readings against distance, a tracing is obtained consisting of one or more gaussian curves. If more than one hemoglobin is present, the tracing may consist of two or more well-defined peaks (Fig. 7) or an extra component may appear as a distortion of a major peak (Fig. 8). In either event gaussian curves are completed for each component, and the areas under each peak are measured by means of a planimeter, as is done for similar tracings of serum proteins. The results may be expressed in relative percentages by dividing the area under each peak by the total area of all peaks combined and multiplying this figure by 100.

The relative percentages of various amounts of hemoglobin S added to hemoglobin A as calculated from electrophoretic patterns are in good agreement with the theoretical concentrations (Table 3). Similar studies of mixtures of hemoglobins A and F showed high hemoglobin F values in concentrations below 30 per cent of the total (Table 4).

Table 3. Recovery of Hemoglobin S from Mixtures of Hemoglobins A and S by Electrophoresis on Paper

Hemoglobin	*Theoretical* (%)	*Found from electrophoresis* (%)
A	70	63
S	30	37
A	60	53
S	40	47
A	60	58
S	40	42
A	50	49
S	50	51
A	40	41
S	60	59

Table 4. Comparison of the Results of the 1-Minute Alkali Denaturation Test According to Singer, and the Relative % Obtained from Electrophoresis on Paper of Known Mixtures of Hemoglobins A and F

Hemoglobin F content (%)	*Alkali-resistant hemoglobin* (%)	*Fetal hemoglobin from electrophoresis* (%)
70	69	77
60	56	62
60	60	..
50	50	46
40	40	42
40	40	38
31	29	35
21	21	32
21	22	..
11	12	26
11	11	27

FERROHEMOGLOBIN SOLUBILITY

The low ferrohemoglobin solubility of hemoglobin S distinguishes it readily from hemoglobin D which has a high solubility (see Table 2). The only other hemoglobin known at the present time to have a low ferrohemoglobin solubility is hemoglobin H.

Ferrohemoglobin solubility may be measured by a modification of the method of Itano (15).

Equipment

A Coleman Junior spectrophotometer or its equivalent is used. Cuvettes: Coleman # 6-310, 10 x 75 mm., used with # 6-108 adapter.

Reagents

Phosphate Buffer. Dissolve 16.9 Gm. monobasic potassium phosphate (KH_2PO_4) and 21.7 Gm. dibasic potassium phosphate (K_2HPO_4) *or* 17.7 Gm. dibasic sodium phosphate (Na_2HPO_4) in carbon dioxide–free distilled water. Adjust the volume to 100 ml.

Procedure

Add the following to small test tubes: 1.8 ml. phosphate buffer, 20 mg. (approximately) sodium hydrosulfite, and 0.2 ml. of buffered hemoglobin solution (see under preparation of hemoglobin solutions). Mix and allow to stand for 15 minutes. A precipitate should form within seconds. Filter through Whatman filter paper No. 5, or its equivalent. Measure into the cuvettes: 3.8 ml. phosphate buffer, and 20 mg. (approximately) sodium hydrosulfite. Add 0.2 ml. hemoglobin filtrate. Mix by inverting twice. Measure absorbancy at 415 mμ.

Control

Measure into a graduated cylinder of 25-ml. capacity 20 ml. distilled water. Add 0.1 ml. hemoglobin solution. Rinse the pipet. Mix by inversion. Transfer about 4 ml. of the solution to a cuvette. Measure the absorbancy of the control solution as described for the unknown.

Calculation

$$\text{Solubility } \% = \frac{A \text{ unknown}}{A \text{ control}} \times 100$$

where A is the absorbancy.

Results

The solubility of hemoglobins A and F has been found to be 90 per cent or higher by this method. The solubility of S is very low. Figure 9 shows the slope of absorbancy readings for known mixtures of hemoglobins A and S, according to the method described.

WET SLIDES

In order to determine sickling of the erythrocytes, a drop of a suspension of red blood cells in a freshly prepared solution of sodium hydrosulfite (37) is placed on a microscope slide, covered with a coverslip and examined under a microscope after 15–20 minutes.

A drop of a suspension of red blood cells in saline is placed on a second microscope slide, covered with a cover slip and sealed in with Vaseline or Cello-seal. The slide is examined directly in 24 hours and 48 hours for the presence of sickle cells.

ALKALI RESISTANCE

This test is used to distinguish between fetal or alkali-resistant hemoglobins and others, especially A and G. Battle and Lewis (2) have described a slight increase in alkali resistance in their patients with hemoglobin J trait.

Two excellent methods for the determination of alkali resistance have been described (3, 32). The one most commonly used is that of Singer (32).

CHROMATOGRAPHY

A recently developed method for the identification of hemoglobins is chromatography on ion-exchange resins (13, 22, 26). A simple way for carrying out chromatography in flat Lucite cuvettes has been described by Huisman and Prins (13, 26). Hemoglobins A, S, C, E, and F have been separated in this way (11). The behavior of the other hemoglobins has not yet been described. Fetal hemoglobin migrates faster on the resin than hemoglobin A, thereby producing two completely separated

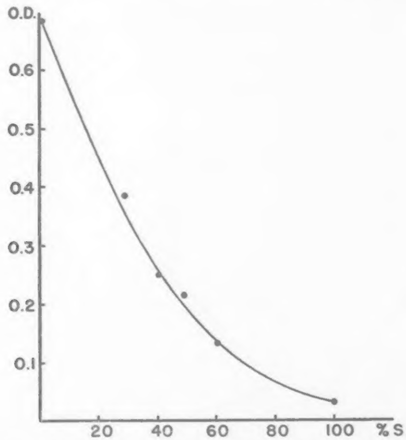

Fig. 9. Relationship of solubility of reduced hemoglobin S to its concentration in mixtures of hemoglobins A and S. O.D. = absorbancy.

zones (Fig. 10). Hemoglobins S and C maintain the same relationship as in electrophoresis at *p*H 8.6 (Fig. 11).

OTHER METHODS FOR THE IDENTIFICATION OF HEMOGLOBINS

Other methods for identifying hemoglobins include salting-out curves, determination of isoelectric points, extinctions at different wavelengths, and determination of the amino acid content of the hemoglobin. These are less practical for routine studies than the methods described.

EXPERIMENTAL

Mixtures of normal adult hemoglobin with various amounts of fetal hemoglobin obtained from cord blood were prepared to correlate the results of the alkali denaturation test with those of electrophoresis. As may be seen, the results of the alkali denaturation test correspond closely to the calculated amounts. However, the electrophoretic results

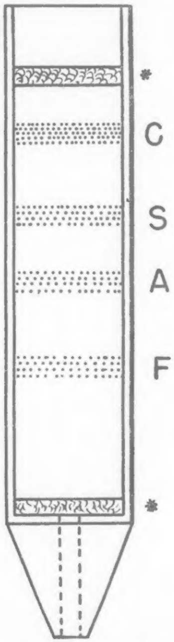

Fig. 11. Chromatography of hemoglobins on ion-exchange resin IRC-50. Schematic diagram.
* = cotton.

are invariably too high, especially in the presence of small amounts of fetal hemoglobin (see Table 3). Better results would probably be obtained from diapositive readings of chromatographic patterns, as suggested by Huisman and Prins (13, 26), since in the chromatographic patterns two completely separated zones are obtained, whereas in the electrophoretic patterns fetal hemoglobin appears as a shoulder on the peak of adult hemoglobin.

Analyses of mixtures of hemoglobins A and S in varying amounts showed good correlation between the ferrohemoglobin solubility, electrophoretic patterns, and the calculated amounts. In calculating the relative percentages of each hemoglobin the entire area under each gaussian curve was measured, excluding the trail. No attempt was made to correct for losses due to trailing. Trailing increases with the age of the sample (23) and when buffers of *p*H 7.6 and less are used (20). Also, as the sample ages, the residue remaining on the application line increases. Presumably this is denatured hemoglobin. The greater amount of residue remaining on the site of application when samples of hemoglobin are suspended in water rather than in buffer of *p*H 8.6 is probably due to the more rapid denaturation of hemoglobin at a lower *p*H. Perhaps the absence of salts contributes. It was particularly noticeable in samples containing abnormal hemoglobins. It was further noticed that electrophoretic patterns of old buffered hemolysates were characterized by increased diffuseness of the hemoglobin zones (see Fig. 6).

Densitometer readings of electrophoretic patterns of patients with sickle cell traits invariably showed minor peaks in the "trail" about halfway between hemoglobin S and the application line. This area is

Table 5. Comparison of Electrophoretic Patterns of 3 Patients with Sickle Cell Trait: Unstained and Stained with Amido Black 10B

			Stained with Amido Black 10B		
Patient	*Hemoglobin*	*Unstained* (%)	S_1[a] (%)	S_2[b] (%)	*Total* (%)
M.B.	A	69	..	..	68
	S	31	24	8	32
S.	A	59	..	..	67
	S	41	29	4	33
B.	A	64	..	..	59
	S	36	19	22	41

[a] S_1 designates the protein peak in the S area closest to hemoglobin A.

[b] S_2 designates the protein peak in the S area closest to the site of application.

indistinguishable by visual inspection from the rest of the trail and its significance, if any, is unknown.

Patterns from three patients with sickle cell traits were stained with Amido Black 10B and readings of these patterns at 600 mμ were compared with the readings of the unstained patterns at 420 mμ (Table 5). In each instance the protein peak in the area of normal adult hemoglobin was narrower than the unstained peak of hemoglobin A. In the area of hemoglobin S, two protein peaks were observed.

DISCUSSION

The identification of abnormal hemoglobins should not depend on electrophoresis at *p*H 8.6 only. Hemoglobins D and S, F and G, J and A, and H and I show similar rates of displacement when subjected to electrophoresis at this *p*H. Furthermore, the rate of migration of hemoglobins A and F at this *p*H is so similar that separation is possible only under the most favorable conditions. Other methods must be used therefore to fully identify the hemoglobins. The distinction between hemoglobins S and D may be made by means of sickling tests and ferrohemoglobin solubility. Hemoglobins F and G differ in resistance to alkali denaturation, hemoglobin F being resistant while G is not. Hemoglobins A and J may be separated by electrophoresis in phosphate buffer at *p*H 7.8. Hemoglobins I and H may be separated by electrophoresis at *p*H 6.5. At this *p*H hemoglobin H migrates anodically while I migrates very slowly toward the cathode. They may be further differentiated by their difference in ferrohemoglobin solubility, that of hemoglobin H being low.

SUMMARY

Practical physical and chemical procedures available for the identification of hemoglobins have been discussed. These include electrophoresis, chromatography, alkali denaturation, and ferrohemoglobin solubility. The conditions essential for satisfactory electrophoretic separations have been considered and two procedures for paper electrophoresis at *p*H 6.5, 7.8, and 8.6 using apparatus for compressed paper strips and for freely suspended horizontal strips are presented. A method for the determination of ferrohemoglobin solubility also has been described.

ADDENDUM

Since this manuscript was submitted for publication, an article has appeared by O. A. Thorup *et al.* (*Science* **123,** 889, 1956) in which a hemoglobin J is described that differs in many respects from the abnormal

hemoglobin described by Battle and Lewis (2) and referred to in the preceding pages. The official designation of the letter J has been given to the abnormal hemoglobin described by Thorup. This hemoglobin is found to migrate electrophoretically between hemoglobins A and I in buffers varying in pH from 6.5 to 8.6. It is not resistant to alkali denaturation, and it has a solubility in reduced form which exceeds that of hemoglobin A.

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Calcium Analyses in Patients Being Treated with EDTA[1]

Marie H. Carr and Hugh A. Frank

PATIENTS ARE BEING TREATED with EDTA in increasing numbers as the result of reports that EDTA aids the mobilization and excretion of calcium from abnormal deposits (1, 2, 3). As a guide to therapy, the director of the clinical laboratory may be asked to estimate not only the calcium concentrations of the serum but also the amounts of calcium excreted in the urine of such patients. The presence of calcium-EDTA complex in the samples to be analyzed creates a technical problem and results in false values unless the samples are specially treated.

One solution to the problem is the destruction of the EDTA by ashing prior to analysis. A simpler approach, one which we have used in analyzing the blood and urine of an EDTA-treated patient, is the acidification of the sample after addition of oxalate. Acetic acid, in the presence of bromcresol green, is used to adjust the *p*H to between 3.0 and 5.0. In this *p*H region, calcium is quantitatively precipitated as its oxalate even in the presence of EDTA (Table 1). Calcium values found in samples containing EDTA, which are treated in this manner, compare favorably with those found in aliquots of the same sample which have been ashed (Table 2). Even though the commonly used procedure for urine calcium as found in Bray's text (4) is designed for the precipitation of calcium oxalate in the presence of acid, the *p*H may be as high as 6.8 and in this case an undetected error of viable magnitude would occur. On the other hand, if the destruction of organic matter in the urine is accomplished according to the method of Shohl and Pedley as recommended by Hawk, Oser, and Summerson (5), the analysis is valid.

From the Research Laboratories, Veterans Administration Hospital, Kansas City, Mo.
Received for publication, May 1, 1956.

[1] EDTA by common usage stands for disodium ethylenediaminetetraäcetate, also known by the trade names Versene or Sequestrene.

Table 1. Effect of *p*H on Calcium Oxalate Precipitation in the Presence of EDTA

	*p*H *After addition of oxalate*	*Ca. recovered (mEq./l.)*	
Untreated sample	5.37	6.83	
Treated with EDTA sufficient to chelate 6.83 mEq./l.	1.5	0.32	Calcium oxalate soluble
	2.0	6.42	
Ca and pH adjusted	3.0	6.80	Usable range
	4.0	6.72	
	4.5	6.55	
	5.0	6.65	
	5.5	6.00	Calcium versenate stable
	6.0	3.74	
	6.7	1.52	
	7.3	0.32	

Table 2. Validity of Analyses of Acidified Samples

Specimen	*Before adding EDTA*		*Usual method of precipitation*		*Acidified precipitation*		*Ashed aliquot*
	*p*H *of sample*	*Ca (mEq./l.)*	*p*H *with oxalate*	*Ca (mEq./l.)*	*p*H *with oxalate*	*Ca (mEq./l.)*	*Ca (mEq./l.)*
Serum of patient treated with EDTA	..	..	7.40	3.63	5.00	4.22	4.36
Urine of patient treated with EDTA	7.45	..	7.40	0.44	5.00	16.92	17.01
Ordinary serum plus EDTA = Ca	7.40	4.66	7.40	0.54	5.00	4.72	4.75
Ordinary urine plus EDTA = Ca	6.25	6.83	5.37	2.93	3 to 5	6.68	6.90

It is important, therefore, not only that the clinical analyst be prepared to meet this problem but that the physician be aware of its existence. Unless the physician notifies his laboratory when a patient is receiving EDTA, the report he receives may be invalid.

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Ultramicro Method for Serum Bilirubin by Diazo Blue Reaction

Ramon E. Stoner and Harry F. Weisberg

PROPER CLINICAL MANAGEMENT of infants with erythroblastosis fetalis necessitates the determination of total serum bilirubin. Of the many modifications of the original diazo reaction on blood serum (1) a micromethod using capillary blood is preferable. One tenth milliliter of serum was used by Davidson *et al.* (2) in their modification of the Ernst and Förster (3) method and by With (4, 5) in his modification of the (alkaline) diazo blue method of Jendrassik and Gróf (7). Hsia *et al.* (8) used 0.05 0.1 ml. of serum in a modification of the Malloy and Evelyn (9) method with the microcell attachment of the Evelyn colorimeter. Caraway and Fanger (10) have recently described a modification of the Malloy and Evelyn method using 0.02 ml. of serum in the Beckman DU spectrophotometer with the microadaptation of Lowry and Bessey (11). The method to be described determines the serum bilirubin on 0.02 to 0.05 ml. of serum using the Coleman Junior spectrophotometer.[1] It is a modification of the (acid) diazo blue reaction used by Thannhauser and Anderson (12) with precipitation of the serum proteins (13, 14). It has been used successfully in our hospital since 1953.

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Presented in part before the Chicago Section, American Association of Clinical Chemists, February 25, 1955.

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The authors wish to thank Dr. Marjorie Sutherland, consulting biostatistician to the Mount Sinai Medical Research Foundation, for the statistical analysis of the data.

Received for publication June 8, 1956.

[1] Coleman Junior spectrophotometer, model 6A, with 12 x 75 mm. round cuvets (Coleman No. 6–308) which require a Coleman (No. 6–106) adapter. The *average* internal diameter of these cuvets is about 1.0 cm. and the *average minimum* volume is 1.5 ml. The cuvets should be checked to be sure that the bottom of the meniscus is above the beam of light.

METHOD

The technic for obtaining a blood specimen by heel or fingertip puncture and the subsequent manipulations are adequately described by Natelson (15), Hsia *et al.* (8), Caraway and Fanger (10), and Kirk (16). These references also give sources of supply for the special pipets, etc. Serum or plasma may be used in the procedure. All chemicals are of analytical reagent grade.

Reagents

Hydrochloric acid, concentrated.

Sulfanilic acid, 0.1% solution. Dissolve 1.0 Gm. of sulfanilic acid in 15 ml. of concentrated HCl and dilute to 1 L. with distilled water.

Sodium nitrite, 0.5% solution. Dissolve 0.5 Gm. of sodium nitrite in distilled water and dilute to 100 ml. Keep in refrigerator. Prepare fresh every 2 weeks.

Diazo reagent. Prepare fresh just prior to using by adding 0.3 ml. of the sodium nitrite solution to 10 ml. of the sulfanilic acid solution.

Ammonium sulfate, saturated solution. Add 80 Gm. of ammonium sulfate to 100 ml. of boiling water; mix until all the crystals have dissolved and cool to room temperature. If crystallization has not occurred, seed with a few crystals of ammonium sulfate. Filter after the excess salt has settled out of solution.

Ethyl alcohol, absolute. Protect against contamination with moisture.

Bilirubin stock standard, 0.2 mg. per ml. Transfer 20.0 mg. of dry bilirubin (Armour) to a 100-ml. volumetric flask. Add chloroform and mix to dissolve bilirubin; dilute to mark with chloroform. Store in refrigerator in brown bottle.[2]

Bilirubin working standard, 0.04 mg. (40 μg.) per ml. Dilute 5.0 ml. of the stock standard with chloroform to the 25-ml. mark of a volumetric flask.

Urea, 5% solution. Dissolve 5 Gm. of urea in distilled water and dilute to 100 ml.

Procedure

A. Total Serum Bilirubin:

1. To a 2.0-ml. volumetric flask (A. H. Thomas No. 5474) add 0.2 ml. of freshly prepared diazo reagent.

[2] Our first working standard was made by diluting the stock standard with absolute ethyl alcohol; this solution was very unstable and had to be used within 10 minutes after preparation. The new working standard, diluted with chloroform, has been found to be stable for up to 1 year when kept in the dark under refrigeration.

2. Using an ultramicro pipet, transfer with rinsing 0.02 ml. (or 0.05 ml.) of serum. Color due to "direct" reacting bilirubin may be noted at this point; if it does appear, *allow for complete color development by waiting for 10 minutes*. If no color develops proceed with next step.

3. Add 0.1 ml. of concentrated HCl and mix.

4. Add 0.2 ml. of saturated $(NH_4)_2SO_4$ and mix.

5. Prepare a "blank" by mixing the following in a 2.0 ml. volumetric flask: 0.2 ml. of diazo reagent, 0.1 ml. of concentrated HCl and 0.2 ml. of saturated $(NH_4)_2SO_4$.

6. Dilute the unknown and the blank to the 2.0 ml. mark with absolute ethyl alcohol. The ethanol should be added down the sides of the flask to avoid disturbing the precipitate and to allow for accurate adjustment of the meniscus.

7. Stopper each flask and shake it vigorously for 1 minute. Leave flasks lying horizontally for 10 minutes.

8. Centrifuge flasks at 2000 rpm for 5 minutes without removing stoppers.

9. Using a bulb pipet, carefully remove a *minimum* of 1.5 ml. of the supernatant, without disturbing the precipitate, and transfer to a round 12 x 75 mm. Coleman cuvet.

10. Adjust cuvet with the blank to 100 per cent transmission at the 580 mμ wavelength. Determine concentration of total bilirubin per 100 ml. of serum from the standard curve. (See below and Fig. 2.)

B. One-minute Direct Reacting Bilirubin:

1. Add 0.2 ml. of freshly prepared diazo reagent to a 2.0-ml. volumetric flask.

2. Using an ultramicro pipet, transfer with rinsing 0.02 ml. (or 0.05 ml.) of serum.

3. Dilute to about 1.5 ml. with 5% urea solution.

4. Add 0.1 ml. of conc. HCl and mix.

5. Prepare a "blank" by mixing the following in a 2.0 ml. volumetric flask: 0.2 ml. of diazo reagent and 0.1 ml. of concentrated HCl.

6. Dilute the unknown and the blank to the 2.0-ml. mark with 5% urea solution.

7. Transfer a *minimum* of 1.5 ml. of the solution to a round 12 x 75 mm. Coleman cuvet.

8. Read 1 minute after addition of serum to diazo reagent (step B-2) with the blank adjusted to 100 per cent transmission at the 580 mμ wavelength. Determine the concentration of the 1-minute direct-reacting

bilirubin per 100 ml. of serum from the standard curve. (See below and Fig. 2.)

C. Preparation of Standard Curve:

1. Prepare bilirubin working standard just before use.
2. In a series of six 2.0-ml. volumetric flasks add approximately 1 ml. of absolute ethyl alcohol.
3. Add respectively 0.0, 0.01, 0.02, 0.05, 0.1, and 0.2 ml. of the working standard (containing 0.0, 0.4, 0.8, 2.0, 4.0, and 8.0 μg. of bilirubin, respectively). The first flask contains the blank.
4. Add 0.2 ml. of diazo reagent and 0.1 ml. of concentrated HCl to each flask and mix. Dilute each flask to mark with absolute ethyl alcohol and mix.[3]
5. After 15 minutes, transfer contents to 12 x 75 mm. round Coleman cuvets and read against the blank set at 100 per cent transmission at 580 mμ wavelength. Repeat readings several times.
6. Plot the observed values on semi-log graph paper (see Fig. 2). The concentration of the bilirubin per 100 ml. of serum is obtained from the formula

$$\text{mg.}/100\text{ ml.} = \frac{\mu\text{g. (from calibration curve)}}{10 \times \text{ml. serum used}}$$

7. Standards should be determined with each batch of unknowns, to check the calibration curve.

RESULTS

Figure 1 illustrates the different absorption maximums for alcoholic solutions of azobilirubin with varying strengths of hydrochloric acid. The curves are expressed on a weight/volume percentage basis to designate the final dilution of the hydrochloric acid added per se and added as part of the diazo reagent. Curves *a*, *b*, and *c* contain 0.03, 0.06, and 1.16 per cent (w/v) hydrochloric acid, respectively. Curve *b* typifies the acid strength of the solution in the Malloy and Evelyn method with the absorption maximum at 530 mμ. Increasing the strength of the acid causes an increased optical density of the solution (decreased percentage transmission) but no shift of the absorption maximum from 530 mμ. Curve *d* (2.26 per cent (w/v) hydrochloric acid) has the strong absorption maximum at 580 mμ typical of the (acid) blue diazo. Additional acid did not increase the

[3] Identical calibration curves were obtained when the standard solutions contained 0.2 ml. of saturated ammonium sulfate.

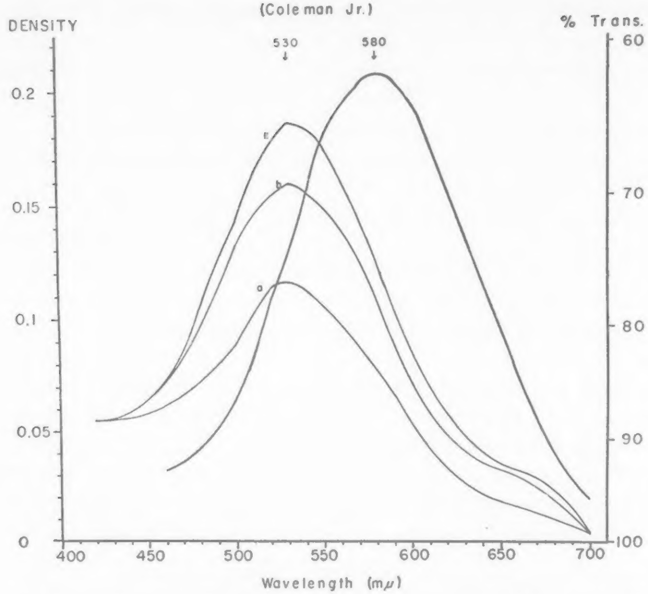

Fig. 1. Absorption of azobilirubin in Coleman Junior spectrophotometer with 12 x 75 mm. round cuvets. Final concentration of HCl (w/v) is 0.03, 0.06, and 1.16 per cent for Curves *a*, *b*, and *c*, respectively; the azobilirubin is red in color and has a maximum absorption at 530 mμ. Curve *d* shows the absorption for the blue diazobilirubin with absorption maximum at 580 mμ; the final concentration of HCl is 2.26 per cent (w/v). A further increase of the acidity did not increase the density of the solution nor shift the absorption maximum.

optical density of the solution nor result in a shift from the 580 mμ absorption maximum.

Figure 2 illustrates the calibration curve obtained using the Coleman Junior spectrophotometer with the 12 x 75 mm. round cuvets (1.5 ml. minimum volume) contrasted to that obtained with the microcell attachment of the Evelyn colorimeter (1.5 ml. of solution placed in the 2.0 ml. open type cell). Use of the microcell attachment of the Evelyn colorimeter results in a steeper curve (increased sensitivity) with the diazo blue reaction.

Venous serums from 4 normal, 4 hemolytic, and 5 obstructive-jaundice patients were obtained from the hospital laboratory. Duplicate determinations by the diazo blue micromethod were made on each serum before adding a known amount of bilirubin (dissolved in chloroform) and also on

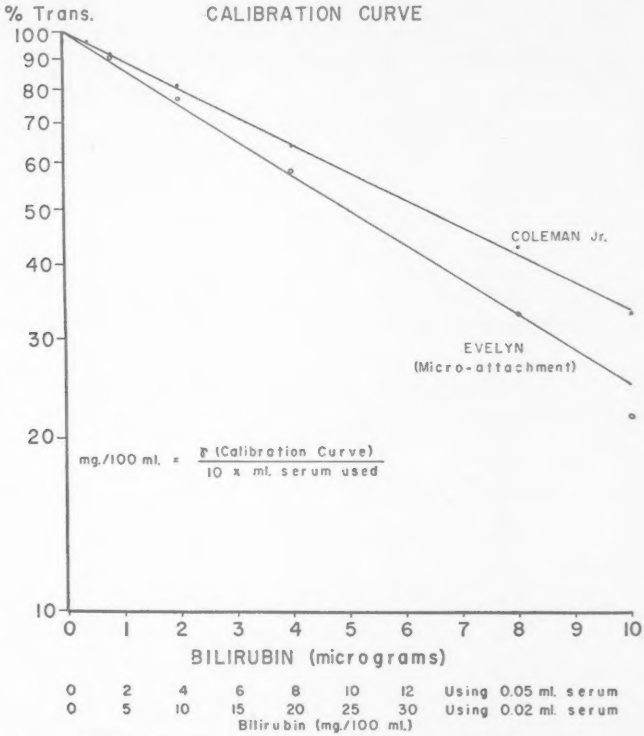

Fig. 2. Calibration curves of blue azobilirubin (final concentration (w/v) of HCl is 2.26 per cent). Upper curve for Coleman Junior spectrophotometer using 12 x 75 mm. round cuvets; lower curve for microcell attachment of Evelyn colorimeter, placing 1.5 ml. of solution into 2.0 mm. open type cell.

the total amount recovered (Table 1 and Fig. 3*A*). Statistical analysis [formulas as in Snedecor (17)] of these data consisted of correlation and regression studies based on the method of least squares. A correlation coefficient of 0.9983 was obtained indicating a strong relationship between the calculated and the actual amounts of bilirubin recovered for these serums. If the calculated and actual recoveries were identical for each serum, the regression line would have a slope of 1.0000; the actual slope was 0.9938 over a range of 0.00 to 7.6 μg. of bilirubin. A t-test value was obtained, $t = 0.356$, indicating that the differences between the two slopes could be due to variations in sampling.

Table 1. Recoveries of Bilirubin from Serum Using the Diazo Blue Micromethod under Routine Conditions

All Values Are Expressed as Micrograms of Bilirubin in the Sample

Venous serum				Bilirubin in sample		
Type	Bilirubin in serum		Bilirubin added	Calculated (C + D)	Determined	
A	Values B	Average C	D	E	Values F	Average G
Normal	0.48				1.34	
	0.58	0.530	0.8	1.320	1.34	1.340
	0.03				2.00	
	0.05	0.040	2.0	2.040	2.40	2.200
	0.00				2.06	
	0.14	0.070	2.0	2.070	2.40	2.230
	0.14				1.75	
	0.30	0.220	2.0	2.220	2.06	1.905
Hemolytic	6.02				7.47	
	6.42	6.220	1.2	7.420	7.47	7.470
	7.47				8.62	
	7.68	7.575	1.2	8.775	8.86	8.740
	1.60				3.29	
	2.13	1.865	1.6	3.465	3.29	3.290
	3.83				5.65	
	4.38	4.135	2.0	6.135	6.40	6.025
Obstructive	1.61				2.42	
	1.75	1.680	0.8	2.480	2.42	2.420
	2.26				3.29	
	2.55	2.405	0.8	3.205	3.45	3.370
	2.84				3.61	
	3.00	2.920	0.8	3.720	3.61	3.610
	4.23				6.33	
	4.68	4.455	2.0	6.455	6.42	6.375
	4.48				6.43	
	4.48	4.480	2.0	6.480	6.43	6.430
Average				4.2919		4.2619

The following regression equation was obtained, using the formula $\hat{Y} = \overline{Y} + b(X - \overline{X})$

Estimated bilirubin recovery = −0.0034 + 0.9938 (Calculated bilirubin)

Because of (a) the correlation coefficient of 0.9983, (b) no significant difference between the averages for the calculated and the actual bilirubin recoveries and (c) no significant difference from 1.0000 for the slope of the regression line calculated from these data, it was concluded that,

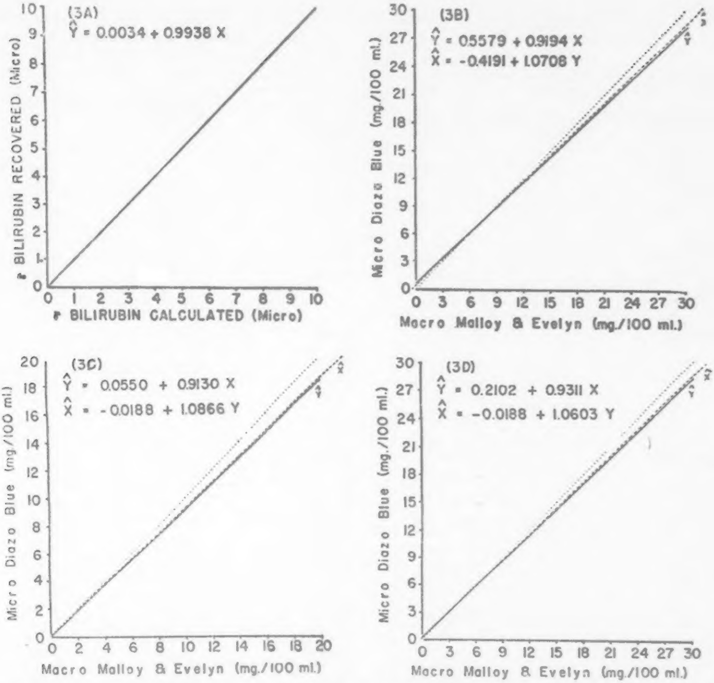

Fig. 3. *A*, actual bilirubin recovered versus calculated recovery. (Because of closeness, the line for the theoretical slope of 1.0000 was omitted.) *B*, *C*, and *D*, Slopes used to estimate values of one method based on test data of the other method, compared to the theoretical of 1.0000 (*dotted line*). *B*, on same sample of venous blood. *C*, Venous blood (Malloy and Evelyn) versus capillary blood (Diazo blue). *D*, Combined data of *B* and *C*.

within the limits of experimental error, 1 μg. of bilirubin would be recovered for each microgram of the calculated value for a specific serum in this series of analyses.

Analysis of variance technics (17) were used to evaluate the significance of variation among bilirubin determinations from different serums, not accounted for by the regression line. The F test provided further evidence of the reliability of the diazo blue micromethod as $F = 1.08$. Since an F value of 1.08 is well below the 2.63 required for the variation to be significant at the 5 per cent level of probability, it was concluded that the unexplained variation in bilirubin recovered from different serums was not significantly larger than the variation among duplicate determinations on the same serum.

Table 2 shows the averages of duplicate bilirubin determinations ob-

Table 2. Comparison of Total Bilirubin Values (Averages of Duplicates) Determined by Malloy and Evelyn Macromethod and by the Ultramicro Diazo Blue Method

Type of serum	*Venous serum*		*Venous serum Malloy & Evelyn (macro)*	*F. T. serum Diazo blue (micro)*
	Malloy & Evelyn (macro)	*Diazo blue (micro)*		
A	*B*[a]	*C*[a]	*D*[b]	*E*[b]
Normal	0.30	0.60	1.40	1.20
	0.20	0.10	0.60	0.60
	0.10	0.10	1.80	1.60
	0.05	0.04	0.70	0.60
	0.00[c]	0.50	1.10	1.60
			0.60	1.00
Hemolytic	20.40	18.40	..	..
	11.40	10.45	..	..
	11.20	9.95	..	..
	9.60	9.65	..	..
	2.40	3.80	..	..
Obstructive	15.60	15.90	2.00	1.90
	17.80	19.40	6.70	4.95
	28.65	29.00	3.30	2.55
	22.90	21.40	15.80	13.65
	28.70	25.30	5.60	4.50
	22.20	19.00	9.20	9.85
	10.00	9.65	4.00	4.45
	9.30	10.10	11.90	12.30
	16.80	15.65	16.00	13.75
	7.90	8.50	3.20	2.65
	6.00	5.90	3.00	2.55
			6.10	6.20
Average	11.50	11.13	5.17	4.77

All values are expressed as mg. per 100 ml.

[a] First series (columns B and C) performed on same sample of venous blood.

[b] Second series (columns D and E) compares results from venous versus capillary (fingertip) blood serum.

([c] Malloy and Evelyn blank more cloudy than usual; result, therefore, was zero.)

tained in two subsequent studies. The first series (columns B and C) gives a comparison of the Malloy and Evelyn macromethod with the diazo blue micromethod, the determinations being done on the same sample of venous blood. The second study (columns D and E) compares the Malloy and Evelyn macromethod determined on serum from blood obtained by venipuncture and the diazo blue micromethod determined on serum from capillary blood obtained by fingertip puncture.

For simplicity of interpretation and comparison, the statistics discussed in relation to Table 1 are shown in column B of Table 3. Those data listed in columns C and D of Table 3 were obtained from the two series

Table 3. Summary of Statistical Analyses

A Correlation and regression	*B* X = Diazo blue calculated; Y = Diazo blue recovered	*C* X = Malloy and Evelyn, venous; Y = Diazo blue, venous	*D* X = M and E[3] venous; Y = Diazo blue, fingertip	*E* X = M and E[3] (columns C and D); Y = Diazo blue (columns C and D)
Correlation coefficient (r)	0.9983	0.9922	0.9960	0.9936
Slope of regression line ($b_{y.x}$)	0.9938			
Diazo blue estimated from Malloy and Evelyn ($b_{y.x}$)		0.9194	0.9130	0.9311
Standard error of regression slope (S_b)	0.0174	0.0286	0.0223	0.0186
$t = \frac{b_{y.x} - \beta}{S_b}$ $\beta = 1.0000$	0.356	2.818[a]	3.901[b]	3.704[b]
$\beta = 0.9194$				0.6290
$\beta = 0.9130$				0.9130
Malloy and Evelyn estimated from diazo blue ($b_{x.y}$)		1.0708	1.0866	1.0603
$t = \frac{b_{x.y} - \beta}{S_b}$ $\beta = 1.0000$		2.476[a]	3.883[b]	3.242[b]
$\beta = 1.0708$				1.435
$\beta = 1.0866$				0.572

Analysis of variance								
Source of variation	MS[1]	DF[2]	MS	DF	MS	DF	MS	DF
Malloy and Evelyn								
Among serums independent of regression			2.9107	19	0.4118	16	1.7356	37
Between duplicate analyses			0.4014	21	0.0800	18	0.2531	39
F			7.25[b]		5.15[b]		6.86[b]	
Diazo blue								
Among serums independent of regression	0.0426	11	2.4992	19	0.3460	16	1.5652	37
Between duplicate analyses	0.0394	13	0.2279	21	0.2633	18	0.2442	39
F	1.08		10.97[b]		1.31		6.44[b]	

[a] Significant at 5 per cent level of probability.
[b] Significant at 1 per cent level of probability.
[1] Mean square.
[2] Degrees of freedom.
[3] Malloy and Evelyn.

of data presented in Table 2; column E lists the results obtained by combining all the data in Table 2 (or columns C and D of Table 3). Table 3 emphasizes the uniformity of results obtained from the three independent studies (columns B, C, and D) and from the combination of C and D (column E). All of the four correlation coefficients are greater than 0.9920, indicating the close agreement between determinations by the Malloy and Evelyn macromethod and the diazo blue micromethod.

Columns C, D, and E of Table 3 show the uniformity of the slopes of regression lines (b = 0.9194, 0.9130, and 0.9311, respectively) used to estimate the micromethod result from the Malloy and Evelyn macromethod test data. The corresponding slopes used to estimate the Malloy and Evelyn result from the diazo blue micromethod test data were b = 1.0708, 1.0866, and 1.0603 (see Fig. 3*B*, *C*, and *D*). Each of these slopes was significantly different from the theoretical value of 1.0000, when tested by the t test. The slopes obtained by combining the two series of data (0.9311 and 1.0603), however, were not significantly different from the slopes obtained for either of the two series in Table 2. This suggests that the diazo blue micromethod may be used with either serum obtained from a vein or serum obtained from a capillary (fingertip puncture) without markedly influencing the results.

The F test (as in column B, Table 3) was used to evaluate the variation among different serums, independent of the regression lines, for the Malloy and Evelyn macromethod and for the diazo blue micromethod. The data in column C were obtained before those in column D. The relatively large and significant mean squares in column C in contrast to the smaller values in column D could be explained on the basis of increased experience and familiarity in preparing the samples and performing the analyses.

DISCUSSION

In van den Bergh's adaptation of the Ehrlich diazo reaction, bilirubin is coupled with diazotized sulfanilic acid resulting in a mixture of bilirubin-azobenzene-sulfonic acid pigments. The mixture, conveniently but erroneously referred to as azobilirubin, has not been separated into its component parts (18). Azobilirubin has indicator properties; it is red in neutral solutions and in acid solutions of *p*H 2.0 to 5.5 (18) with an absorption maximum at 530 mμ (19). Azobilirubin is blue in strongly acid solutions with a very strong absorption at 580 mμ; it is also blue ("green") in alkaline solutions but the alkaline form of diazo blue is not very stable (20). It is evident (Fig. 1) that the absorption of the blue azobilirubin micromethod (Curve *d*) is much stronger than that of the red azobilirubin curve obtained by the Malloy and Evelyn macromethod (Curve *b*). Malloy and Evelyn (9) were cognizant of the color's being sensitive to *p*H changes but felt that the presence of serum protein provided "a buffer substrate sufficient to stabilize the pH-sensitive color of the azobilirubin." The diazo blue method is used without a serum blank; the turbidity due to lipids is removed by the precipitation of the proteins.

The microcell attachment of the Evelyn colorimeter was used by Hsia *et al.* (8) and Caraway and Fanger (10) have used the micro-adapter for the Beckman DU spectrophotometer. We feel that the Coleman is more widely distributed and is easier for the average analyst to use. An attempt was made to use the Hsia method with the Coleman spectrophotometer but the calibration curve was too flat (sensitivity represented by Curve *b* in Fig. 1). Utilization of the diazo blue reaction (Curve *d* of Fig. 1) shows an increased sensitivity when compared to the diazo red reaction used in the Malloy and Evelyn modifications. Figure 2 illustrates that the Evelyn microattachment (using a volume of 1.5 ml.) has a greater sensitivity with the diazo blue reaction than the Coleman Junior spectrophotometer (with the 12 x 75 mm. round cuvet). The Coleman 10 x 75 mm. round cuvets have an internal diameter of about 0.8 cm. and require an average *minimum* volume of 1.0 ml.; we found that the approximately 25 per cent longer light path of the 12 x 75 mm. cuvet (internal diameter about 1.0 cm. and average *minimum* volume of 1.5 ml.) gave increased sensitivity with better reproducibility, advantages that compensated for the necessary additional 0.5 ml. of solution.[4]

Diluting a specimen will alter its acidity unless a buffer solution is used for the dilution (14); thus the sensitivity of the reading will be decreased (e.g., from Curve *b* to Curve *a* in Fig. 1). Malloy and Evelyn (9) recommended diluting their final dilution if the bilirubin level exceeded 15 mg. per 100 ml. of plasma. Gray and Whidbourne (21, 22) reported that the proportionality between the color density and the concentration of (red) azobilirubin did not hold at high concentrations. The calibration curves (Fig. 2) show a good proportionality for bilirubin levels up to 20 or 50 mg. per 100 ml. when 0.05 or 0.02 ml. of serum are used, respectively, with the diazo blue method.

When alcohol is added to hasten the reaction of bilirubin with diazotized sulfanilic acid in the van den Bergh reaction, precipitation of the plasma proteins results with the possibility that some bilirubin or azobilirubin (especially the "direct" bilirubin (23)) may be lost in the protein precipitate. Van den Bergh and Grotepass (24) tried to overcome this difficulty by precipitating the protein from an alkaline solution before adding the diazo reagent but did not have too much success. Thannhauser and Anderson (12) tried protein precipitation, after the diazo reacted, by adding alcohol and ammonium sulfate; some losses of bilirubin

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[4] Depending upon the internal diameter of the cuvet, the minimum volume needed for the bottom of the meniscus to clear the beam of light will *vary* from 0.9 to 1.1 ml. and 1.4 to 1.7 ml. for the 10 x 75 mm. and 12 x 75 mm. cuvets, respectively.

Table 4. COMPARISON OF METHODS USING ALCOHOL IN DETERMINATION OF TOTAL SERUM BILIRUBIN

Method	*Amount of serum used* (ml.)	*Final volume of solution* (ml.)	*Final serum dilution*	*Final alcohol percentage* (v/v)	*Final ammonium sulfate dilution*
Stoner & Weisberg	0.02	2.0	1:100	74[a]	1:10
	0.05	2.0	1: 40	72.5[a]	1:10
King & Coxon[16]	0.1	1.0	1: 10	68[a]	1:20
	1.0	10.0	1: 10	68[a]	1:20
Malloy & Evelyn[22]	1.0	10.0	1: 25	50[b]	..
Hsia *et al.*[12]	0.05	1.3	1: 52	46[b]	..
	0.1	1.3	1: 26	46[b]	..
Caraway and Fanger[1]	0.02	0.22	1: 24	45[b]	..

[a] Ethanol.
[b] Methanol.

were still noted and occasionally the supernatant solution was not clear with difficulty in colorimetry. The methods of Jendrassik and Czike (29) and Haslewood and King (13) also suffer from the loss of bilirubin in the protein precipitate. King and Coxon (14), however, report precipitation of protein by ethanol and ammonium sulfate without loss of bilirubin.

Methods have been evolved to allow coupling of the bilirubin without precipitation of the proteins; these are exemplified by the methods of Malloy and Evelyn (9), Rappaport and Eichhorn (25), Jendrassik and Cleghorn (26), Powell (27) and Gray and Whidbourne (21, 22). The Malloy and Evelyn (9) method [and its modifications (8, 10)] is based on the addition of sufficient methyl alcohol to allow coupling but still insufficient (50 per cent) to precipitate the proteins from the serum, the final dilution being 1:25 (Table 4). This diluted serum results in a very pale red color, the use of methanol results in occasional cloudy filtrates (due to alcohol precipitation of the protein?), and the serum blank may be turbid or opalescent due to lipids, with resulting inaccuracies in low bilirubin concentrations.

The nonalcohol coupling methods (21, 22, 25, 26, 27) utilize caffeine (and other compounds) and are better for low concentrations of bilirubin but frequently result in impure colors (14). Such methods, however, require anywhere from 6 to 24 hours for complete coupling of the "indirect" bilirubin in cases of hemolytic jaundice (18, 21); this has also been our experience. Since our interest was in patients with erythroblastosis fetalis, our attention reverted to an "alcohol" method for total serum bilirubin. We did not pay as much attention to the 1-minute prompt direct-reacting fraction which is not considered to be a separate type of

bilirubin (22, 28, 30). However, a procedure is described for the determination of the one-minute direct fraction using an additional 0.02 or 0.05 ml. of serum; the use of a separate sample avoids a change in acid strength by dilution with alcohol.

We found clearer filtrates (supernatants) when the ethanol was added down the sides of the flask (step A-6 of PROCEDURE) in contrast to methanol; ethanol is a better protein precipitant. Cloudiness will be avoided if the absolute ethyl alcohol is protected so that it is not diluted with moisture from the atmosphere. Complete precipitation of the proteins has been achieved with the new alcohol concentration (approximately 75% final dilution) and the use of saturated ammonium sulfate in a final dilution of 1:10 (Table 4); the supernatant solution is always clear. A frequent loss of "direct" bilirubin on precipitated protein seen in cases of regurgitation (obstructive) jaundice is avoided by our procedure. It is extremely important that the 10-minute wait (step A-2 of PROCEDURE) be observed in the presence of a "direct" reaction, before adding the concentrated HCl and saturated ammonium sulfate; if the described procedure is followed, any loss of bilirubin will be minimal and not interfere with the use of this method.

The ultramicro diazo blue method for total serum bilirubin is extremely useful in following the progress of patients with erythroblastosis fetalis. The method is relatively simple and has been used by our hospital technicians without any difficulty.

SUMMARY AND CONCLUSIONS

An ultramicromethod is presented for the determination of serum bilirubin by the (acid) diazo blue reaction, using heel puncture or fingertip blood. Diazotization is accomplished in a strongly acid medium with the resulting blue azobilirubin having a maximum absorption at 580 mμ. The method is extremely reproducible and sensitive, being carried out in final serum dilutions of 1:100 to 1:40. The method is described for use with the Coleman Junior spectrophotometer and can be adapted to other instruments.

The diazo blue micromethod compares very favorably with the Malloy and Evelyn method which was done in parallel. The results of this study also suggest that the diazo blue micromethod can be used on venous serum or serum obtained from fingertip puncture without having markedly altered results.

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Measurement of the Electrical Conductivity of Sweat

Its Application to the Study of Cystic Fibrosis of the Pancreas

Truman S. Licht, Milton Stern, and Harry Shwachman

THE ELECTROLYTE CONTENT of thermally induced sweat has been the subject of numerous investigations (1, 2, 3). Reported variation in the composition of eccrine sweat obtained from healthy subjects may be attributed to many factors, such as duration of sweat period, rate of sweating, humidity, environmental temperature, skin temperature, state of hydration, acclimatization, salt intake, type of heat stimulus, method of collection, and the reliability of analytical procedures. The solid content of sweat varies from 0.5 to 1.0 per cent and is about equally divided between the organic and inorganic constituents. The basic composition of healthy eccrine sweat is summarized in Table 1. (4, 5a.).

There are a limited number of pathologic states, such as adrenal dysfunction (6, 7) and cystic fibrosis of the pancreas, in which significant alterations of sweat composition have been noted. In cystic fibrosis of the pancreas, the abnormalities are of such a great magnitude that the measurement of the electrolyte content of the induced sweat has been used by us and others as a diagnostic procedure for this disease. The original demonstration of the elevation of the sodium, chloride, and potassium concentrations in this disease was given in a series of reports by di Sant'Agnese *et al.* (8–11). In a study involving over 1000 infants and children, both healthy and ill with a variety of conditions, we have not en-

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This work was in part supported by the Sharples Fund and in part by a grant from the National Cystic Fibrosis Research Foundation and by Research Grant A-80 from the National Institute of Arthritis and Metabolic Disease of the National Institutes of Health, U. S. Public Health Service.

The helpful contributions of Professor A. J. deBethune of Boston College and Dr. A. Steinberg of the Children's Medical Center are gratefully acknowledged.

Received for publication June 8, 1956.

Table 1. Basic Composition of Sweat Obtained from Healthy Subjects

Inorganic constituents		*Organic constituents*	
Sodium	15–60 mEq./L.	Lactic acid	17–330 mEq./L.
Potassium	3–8 mEq/.L.	Urea	3–7 mEq./L.
Chloride	15–60 mEq./L.	Amino acids (as nitrogen)	0.7–1.5 mEq./L.
Calcium	0.1–1.0 mEq./L.		
Sulfate	0.4–1.8 mEq./L.		
		Other constituents	
Iron, magnesium, Copper, phosphate, Iodide, fluoride	μg. quantities	*pH*	4.5–7.1

countered any instance in which comparable elevated values for the sodium and chloride in thermally induced sweat are found (12).

The present investigation dealing with the electrical conductivity of sweat solutions was undertaken to obtain further information concerning the properties of thermally induced sweat of patients with cystic fibrosis of the pancreas, in miscellaneous illnesses, and in healthy individuals. The electrical conductivity measurement is practical and highly sensitive. The procedure of collecting sweat samples and the method of measuring electrical conductance of sweat are described. The findings for 124 subjects are presented.

The observed electrical conductance of an electrolyte solution is a function of several variables: the species of ions present in the solution, the concentration of the ions, the temperature, and the geometry of the conductivity cell. If a constant temperature and a given conductivity cell are employed, the conductance[1] becomes a measure of the kind and concentration of the ions present.

Modern electronic conductance bridges, available at moderate cost, make measurement of solution conductance simple and rapid with an accuracy of better than 1 per cent. The solution is not consumed or affected by the measurements and may be used for further studies. Excellent reviews of conductance theory (13, 14a, 15) and applications to analytical problems (16a, 17) are available.

Application of conductivity principles to quantitative analysis of sweat solutions has been limited probably because of the difficulty of interpretation of results when more than one electrolyte is present in solution. Lundgren *et al.* (18) studied sweat obtained from arm bag col-

[1] The conductivity, which is also referred to as the specific conductance, is obtained by multiplying the observed conductance by the cell constant. A convenient unit of conductivity is the micromho per cm. (μmho/cm.) which is defined as:

$$1\ \mu\text{mho/cm.} = 10^{-6}\text{ohm}^{-1}\text{cm.}^{-1}.$$

lections of 12 healthy adult males and noted a high correlation between the sodium and chloride content of sweat and the specific electrical conductivity. They obtained conductivity values ranging from 2000 to 11,000 micromhos/cm. and noted that the conductance data were not obtained at constant temperature. LeVeen (19) has reported a micromethod for the determination of the electrical conductivity of drug-induced sweat.

EXPERIMENTAL

Apparatus

Conductivity Bridge. Serfass Conductance Bridge, Model RCM 15, A. H. Thomas Co., Philadelphia, Pa.

Conductivity Cell. This cell, shown in Fig. 1, was constructed to permit measurement of small volumes of solution (about 3 ml.). The electrodes were platinized with 3% platinic chloride and were covered with distilled water when not in use.

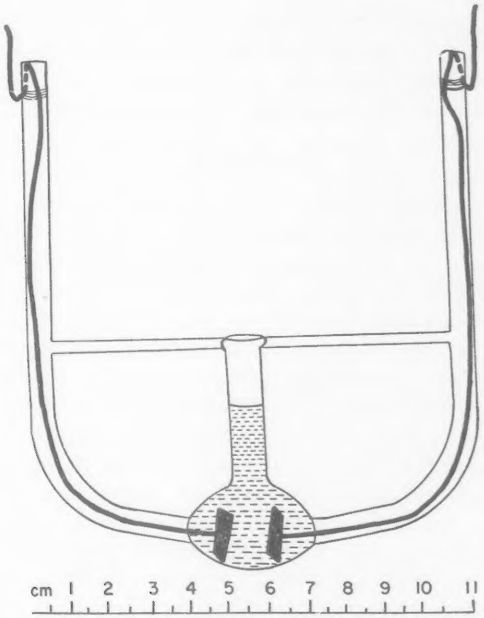

Fig. 1. Conductivity cell.

Constant Temperature Bath. Maintained at 25.0 ± 0.5°.

Sweat Suit. Made of plastic which fits firmly around neck and encloses the entire body.

Gauze Pads. Curity Brand 3″ × 3″ 12-ply gauze pads selected to give minimal "blank" values.

Reagents

Potassium Chloride Standard Solutions

Stock standard, 0.1000 N

Diluted standards, 10.00 mEq./L. (i.e. 0.01000 N) 2.50, 2.00, 1.50, 1.00, and 0.500 mEq./L.

Sodium Chloride Standard Solutions

Stock standard, 0.1000 N

Diluted standards, 2.50, 2.00, 1.50, 1.00, and 0.500 mEq./L.

Mixed Standards

1.00 mEq./L. of potassium chloride with 2.50, 2.00, 1.50, 1.00, and 0.50 mEq./L. of sodium chloride

Distilled water specific conductance not greater than 2 micromhos/cm.

Procedure

Collection of Sweat Sample. This procedure has been described by Shwachman *et al.* (21). The midback of the subject is washed with distilled water and dried. A 3-inch square 12-ply gauze pad is weighed to the nearest milligram in a stoppered, dry, 125-ml. Erlenmeyer flask. Neither the pad nor the prepared area of the back should be touched with the fingers. Using forceps, the pad is placed on the patient's back and covered with a clean polyethylene sheeting of slightly larger area than the pad. The sheeting is taped to the back with eight strips of adhesive tape, two strips of tape to each side of the sheeting. The patient is then placed in the plastic sweat bag, the bag is closed, and the patient covered with blankets. A sufficient amount of sweat (i.e., approximately 1 Gm.) may be absorbed by the gauze pad in approximately 45 minutes, although in some instances it may take 20 minutes and in other cases as long as 2 hours. When the collection period is over the patient is removed from the bag and the sweat-soaked gauze pad is replaced (using forceps) in the

stoppered Erlenmeyer flask. The flask is reweighed and the difference between the two weights represents the quantity of sweat collected.

Measurement of Conductance. Twenty milliliters of distilled water are added to the Erlenmeyer flask, which is thoroughly swirled to elute the electrolyte from the pad. The resulting solution will henceforth be referred to as the "sweat solution."

The conductivity cell is rinsed with at least three successive portions of distilled water and two 1-ml. portions of the sweat solution. The rinsings are discarded. The cell is filled to a level above the electrodes with the sweat solution and the cell is placed in the constant temperature bath at 25.0° for at least 5 minutes. The cell is removed from the bath, wiped dry, and the conductance measured. When using the Serfass conductivity bridge, the cell constant compensator should be set at 1.000 unless the actual cell constant is in the range of the compensator, 0.900 to 1.100.

Evaluation of Blank. The solution for measuring the conductivity of the blank was obtained by placing one 3″ × 3″ gauze pad in a dry Erlenmeyer flask, adding 20.0 ml. of distilled water and swirling thoroughly. An average acceptable blank gave a specific conductance of approximately 30 micromhos/cm. Inasmuch as the distilled water was not considered acceptable if its specific conductance was greater than 2 micromhos/cm., the bulk of the impurities came from the gauze pads. It was found necessary to examine each package of gauze pads (100 pads per package) by determining the blank value for each of three pads selected from the top, middle, and bottom of the package, respectively. If any one of the pads gave a blank for the specific conductance of greater than 50 micromhos/cm. the lot was not considered usable.

Calculations. The sweat conductivity, L_s, may be calculated from the relationship:

$$L_s = (J_o - J_b)KF \qquad (1)$$

where J_o = observed conductance of sweat solution in micromhos at 25.0°.

J_b = observed conductance of blank solution in micromhos at 25.0°.

K = cell constant in cm.$^{-1}$

F = (W + V)/W, dilution factor

W = weight of the sweat sample in grams

V = volume of distilled water added in milliliters

RESULTS

Cell Constant

In order to permit comparison of conductance data obtained with different cells, the cell constant, K, must be known. This constant is determined by the equation

$$K = \frac{L}{J} \tag{2}$$

where L and J are the specific and observed conductances, respectively, for a standard solution. Table 2 shows evaluation of the cell constant used throughout this investigation.

Conductivity Concentration Relationships for Single Electrolytes

The observed conductance of a single electrolyte in solution is an empirical function of concentration, if the temperature and cell characteristics are constant. This function is frequently linear over a limited range. The principal electrolytes of interest in this investigation are sodium chloride and potassium chloride. The conductance of single electrolyte solutions of sodium chloride or potassium chloride, prepared in comparable concentration ranges as the diluted sweat samples, are given in Fig. 2. The slope of the straight lines define a quantity called the equivalent conductance. The observed slopes of the sodium and potassium chloride lines respectively are 121 and 146. These agree well with the theoretical equivalent conductances of 123–124 and 146–148 respectively as given by MacDougall (14b).

Conductivity of Mixtures

The conductivity of a multicomponent electrolyte solution is equal to the sum of the conductivities of its individual ions (16b). Results of conductance measurements for mixtures of sodium and potassium chloride, again in the approximate range of the diluted sweat solutions, are given in Table 3.

Calculated conductivities of the mixture, $L_{NaCl} + L_{KCl}$, based upon the

Table 2. Evaluation of Cell Constant (25.0°C)

KCl Concentration	*Specific conductance, L. (14c)*	*Observed conductance, J*	*Cell constant, K*
0.1000N	12,890 micromhos/cm.	16,800 micromhos	0.7673 cm.$^{-1}$
0.01000N	1,411	1,860	0.7586
Average			0.7630

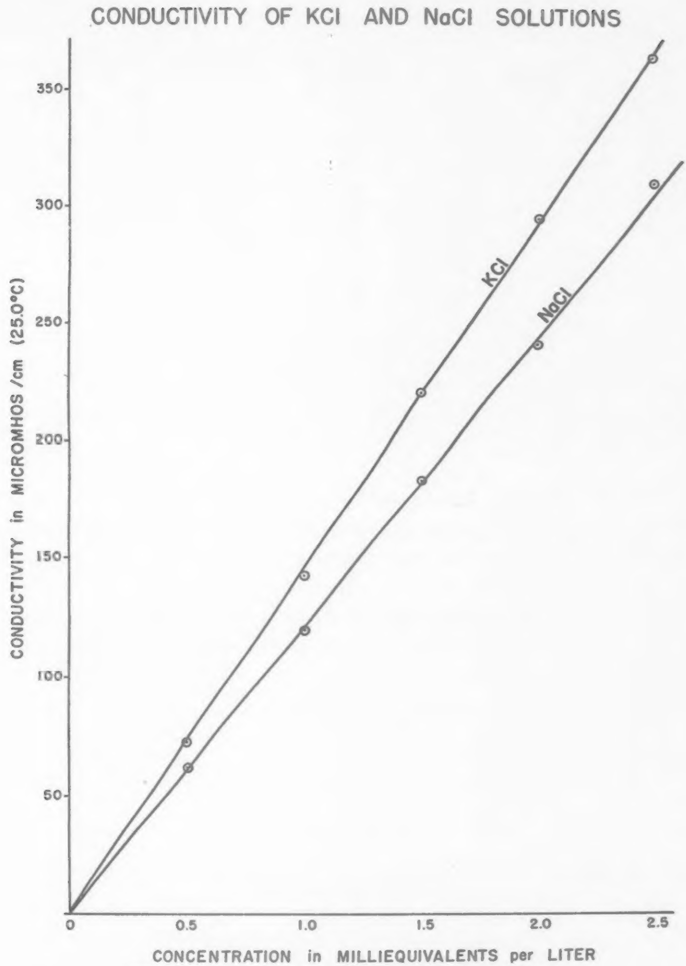

Fig. 2. Conductivity of KCl and NaCl solutions.

conductivity values for the pure components given in Fig. 2 may be obtained from the following relationship:

$$L_{NaCl} + L_{KCl} = 121\ (NaCl) + 146\ (KCl)\mu mhos/cm.\ (25.0^\circ) \qquad (3)$$

where (NaCl) and (KCl) indicate the concentration of the sodium and potassium chloride solutions in milliequivalents per liter. The agreement

Table 3. CONDUCTANCE OF MIXTURES (25.0°)

NaCl (mEq./l.)	*KCl* (mEq./l.)	*Conductivity (experimental)* (μmhos/cm.)	*Conductivity (calculated)* (μmhos/cm.)	*Relative difference* (%)
0.50	1.00	205	207	−1.0
1.00	1.00	268	267	+0.4
1.50	1.00	318	328	−3.1
2.00	1.00	387	388	−0.3
2.50	1.00	445	449	−0.9
3.00	1.00	512	509	+0.6
3.50	1.00	570	570	0.0
4.00	1.00	629	630	−0.2
4.50	1.00	691	691	0.0
5.00	1.00	749	751	−0.3

between the observed and calculated values for the conductivities of these mixtures is better than 1 per cent, which is again the magnitude of the experimental error.

Conductivity of Sweat

The conductivity of sweat collected from 124 subjects was measured by the method outlined in this paper. Forty-seven of this group were

Table 4. TYPICAL SWEAT CONDUCTIVITY DATA

Subject #	*Sample Size, W* (grams)	*Dilution factor, F*	*Conductance* $J_o - J_b$ (μmhos)	*Sweat conductivity,* L_s (μmhos/cm.)
A. FOR CHILDREN WITH CYSTIC FIBROSIS				
474	0.4308	47.43	300	10,860
505	2.0841	10.60	1,670	13,510
532	0.9847	21.31	721	11,720
646	4.5854	5.36	3,480	14,240
654	0.5798	35.49	630	17,060
B. HEALTHY CHILDREN AND THOSE WITH MISCELLANEOUS DISEASES (CONTROL GROUP)				
513	1.0230	20.55	282	4,420
520	1.9080	11.48	625	5,480
526	1.6731	12.95	375	3,710
650	0.3528	57.69	135	5,940
651	5.4367	4.68	960	3,430

C. SUMMARY OF SWEAT CONDUCTIVITY DATA

Group	*No. in group*	*Sweat conductivity* (μmhos/cm.)	
		Mean	*Range*
Cystic fibrosis	47	16,150; S.D. = 3,500	9,000–24,640
Control group	77	5,560; S.D. = 2,360	2,080–14,380

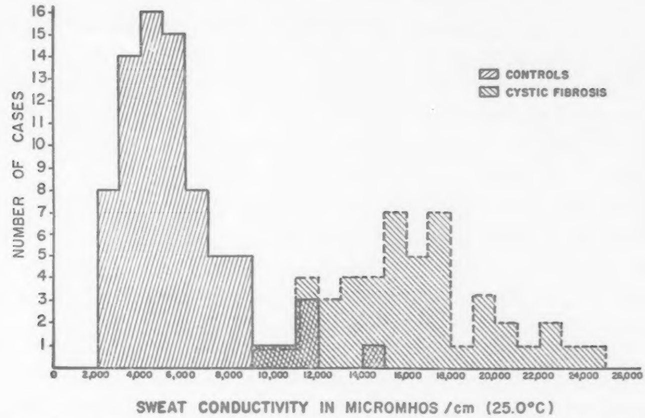

Fig. 3. Sweat conductivity: Frequency distribution 77 controls and 47 patients with cystic fibrosis.

patients with cystic fibrosis. Of the remaining number in this study, 6 were healthy and 71 had a variety of illnesses. The age range of the entire group was four weeks to sixteen years.

Table 4 lists typical data from 10 subjects. These ten subjects have been divided into two groups—those having cystic fibrosis and those having miscellaneous illnesses. Table 4 also gives the mean values and ranges for these two groups. The mean sweat conductivity of the cystic fibrosis group (16,150; S.D. = 3,500 μmhos/cm.) is nearly three times as large as that of the control group (5560; S.D. = 2360 μmhos/cm.).

Figure 3 illustrates the frequency distribution and range of sweat conductivity values encompassed by the two groups studied.

DISCUSSION

The application of the conductivity measurement of thermal sweat provides a diagnostic tool in the laboratory investigation of patients suspected of having cystic fibrosis of the pancreas. It can be seen from Fig. 3 and Table 4-C that the cystic fibrosis patients have a sweat conductivity which is three times the mean for the control group. There are six patients (8 per cent) in the control group with values above 9000 μmhos/cm. which is the lowest value encountered in the cystic fibrosis group. It is logical to assume the conductivity elevation in the cystic fibrosis patients is principally due to the increased sodium chloride content. The extent of the contribution of other electrolytes to the total conductivity may vary and will require further study.

The findings for our control group of children correspond with the results, previously cited, of Lundgren (18) for healthy adults.

The importance of determining the conductance of the sweat samples at a constant temperature is indicated by an approximate increase of 2 per cent per degree rise in temperature of the solution (16c). In this study the temperature was kept constant at 25.0 ± 0.5°.

Other effects to be considered are the dilution of the original sample and the sweat density. The dilution factor, F, of Equation 1 is calculated from:

$$F = (W + V)/W \tag{4}$$

where W is the weight of the sample in grams and V is the volume in milliliters of distilled water added in the original dilution. Strictly speaking, Equation 4 is not exact unless the volume of the sweat sample in milliliters is substituted for its weight. If the density were unity, 1 Gm. of sweat would have a volume of 1 ml. The density of normal profusely secreted sweat has been reported by Rothman (5b) to be in the range of 1.001–1.005 Gm. per ml. The density of sodium chloride solutions up to 170 mEq./L. (20a), of potassium chloride solutions up to 135 mEq./L. (20b) and of pure water (20c) all lie within 0.997 to 1.005 Gm. per ml. in the temperature range of 20–25°. Thus it would appear that the relative error in the dilution factor, F, as defined in this paper, is not greater than ±0.5%.

The sweat conductivity measurements were made on samples of varying weights diluted with 20 ml. of distilled water. The final dilution ranged from a five-fold one to slightly over a fifty-fold dilution, although in most cases the dilution was less than twenty-five-fold. The conductance-concentration relationship is linear only over limited ranges. Therefore calculations of sweat conductivity based on measurements of dilute solutions cannot be accurately extrapolated to more concentrated solutions. An example of the magnitude of this error is afforded by the conductance-concentration data of Table 1 for the standard potassium chloride solutions. If the specific conductance of the 0.1N potassium chloride solution were calculated by Equation 1 using the data of Table 1 for the specific conductance of 0.01N potassium chloride solution, then the calculated value would be 10 per cent higher than the true value.

Evaluation of current data and studies now in progress indicate that the measurement of the concentration of chloride and/or sodium in sweat is a more reliable diagnostic index of cystic fibrosis of the pancreas than is the sweat conductance measurement.

SUMMARY

1. A method has been described for the measurement of the electrical conductivity of thermally induced sweat.

2. Conductivity data of sweat samples collected from 124 children ranging in ages from 4 weeks to 16 years is presented. Forty-seven patients have cystic fibrosis of the pancreas and exhibit a mean value for the sweat conductivity of 16,150 micromhos/cm. In the remaining 77 subjects, of which 6 were healthy and the others had miscellaneous illnesses, the mean value for the sweat conductivity is 5,560 micromhos/cm. All of the subjects with sweat conductivities less than 9000 micromhos/cm. did not have cystic fibrosis; all of the subjects with sweat conductivities greater than 12,000 micromhos/cm., with one exception, had cystic fibrosis. The intermediate range (9000 to 12,000 micromhos/cm.) comprised less than 10 per cent of the subjects studied. It has been shown that measurement of chloride and/or sodium concentration in sweat is a more reliable diagnostic index of cystic fibrosis of the pancreas.

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Some of the Variables Involved in the Fractionation of Serum Proteins by Paper Electrophoresis

Richard J. Henry, Orville J. Golub, and Charles Sobel

MANY HUNDREDS OF PAPERS have been published on the fractionation of serum proteins by paper electrophoresis. The results reported by different workers show significant variations in the relative concentrations of the various protein fractions of normal serum. It is evident that the causes are to be found in the numerous variables in the technic involved. The subject of this investigation is a study of what appear to be the major variables involved in a single standardized technic. Data are also given on the stability of nonsterile samples at room and refrigerator temperatures, and on normal values obtained by the standardized technic adopted.

STANDARDIZED PROCEDURE

The apparatus utilized throughout this investigation was the inverted-V form (1), marketed by the Spinco Division of Beckman Instruments, Inc., Belmont, California. The staining technic is that of Jencks, Jetton, and Durrum (2) with the exceptions of the washing procedure (3) and the temperature of heat-fixation. With the exception of the particular variable under study, the "standardized procedure" was followed in strict detail, employing the elution technic for quantitation.

Reagents

Veronal-sodium veronal buffer, pH 8.6, μ = 0.083 (3.12 Gm. veronal + 17.1 Gm. sodium veronal per L.).

Dye solution: to 0.10 Gm. bromphenol blue and 50.0 Gm. $ZnSO_4 \cdot 7H_2O$ add about 25 ml. of 95% ethanol. Mix thoroughly and add 5% (v/v) acetic acid to make 1 L. Mix until complete solution is effected

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From the Bio-Science Laboratories, Los Angeles 64, Calif.
Received for publication August 12, 1956.

Wash solution: 5% (v/v) acetic acid.
Fixative solution: 5% (v/v) acetic acid containing 0.3% sodium acetate ($CH_3COONa \cdot 3H_2O$.)
Eluting solution: approximately 0.01 N NaOH.

Procedure

1. Place adequate buffer in both electrode compartments, place a full complement of 8 paper strips (Whatman 3 MM, Spinco) on the rack and place the rack in the cell.

2. Wet strips thoroughly with buffer, replace cell cover and allow at least 15 minutes for equilibration.

3. Transfer exactly 10 μl. of serum from an ultramicropipet (TD 25 μl., graduated at 5 μl. intervals) to the Spinco striper. The sample is then applied to the paper from the striper.

4. Electrophoresis is carried out at room temperature (approximately 25°) for 16 hours at 80 V. (3.2 V. per cm.) or for 7 hours at 144 V. (5.8 V. per cm.).

5. Heat-fix the strips at 107 ± 3° for 30 minutes in a forced-draft oven.

6. Stain at room temperature for 6 hours or overnight.

7. Wash in 2 changes of wash solution for 6 minutes each.

8. Fix for 6 minutes in the fixative solution.

9. Blot strips between clean absorbent paper (filter paper, hand towels, etc.) and dry at 107° (temperature and time not critical).

10. Demarcate protein components by pencil lines, placing the lines at the point of lightest staining between components. In general, this is about midway between the points of deepest staining. Also mark off a paper blank, its length being about the average of the protein segments. Cut out segments and place in test tubes.

11. Elute dye from each segment for one-half hour with 6 ml. 0.01N NaOH, mixing tube contents three times during this period. By the end of the elution period the strips should be white and show no evidence of retained dye.

12. Read absorbance (A) within next one-half hour at 540 mμ. With a half band-width of 20 mμ or less, no correction for deviation from Beer's law is required.

13. Subtract A_{blank} from each of the A values of the protein components.

14. Calculate the A_{total} and the "% of total protein" for each fraction.

15. If desired, concentration in Gm. per 100 ml. for each fraction can

be calculated if the total serum protein concentration is determined by biuret or other method.

VARIABLES IN THE ELECTROPHORETIC SEPARATION

Paper

Comparison of Whatman Nos. 1 and 3 MM and Schleicher and Schuell No. 598 revealed no significant difference between the two papers of similar weight and thickness—i.e., between Whatman 3 MM and SS 598—but the albumin/globulin ratio (A/G ratio) was consistently about 5 to 10 per cent higher using the thinner Whatman No. 1 paper than with either of the other two papers. This difference proved to be completely accountable for by the difference in degree of albumin "trailing" experimentally determined for the two types of paper. Human albumin (Lot No. 940 C2B, Hyland Laboratories, Los Angeles, Calif.) left a trail twice as great on Whatman 3 MM and SS 598 as on Whatman No. 1. The thinner paper, however, was more difficult to manipulate when wet because of ease of tearing.

For quantities of albumin ranging from 100 to 600 μg., the quantity per unit area remaining behind was constant, thus confirming previous claims (2, 4, 5) that albumin "trailing" is a saturation phenomenon. For the human albumin and Whatman 3 MM paper, this quantity in 36 strips averaged 1.4 μg. of albumin per sq. cm. of paper (corrected for paper blank). With Whatman 3 MM paper 3 cm. wide and a migration distance of 7 cm. from the point of application to the nearest edge of the albumin component, the loss of albumin in the trail for 10 μl. of sera containing 1, 2, 3 and 4% albumin is, therefore, about 30, 15, 10 and 7.5 per cent, respectively. Furthermore, the total globulins would be overestimated by about 30 μg. Jencks *et al.* (2) reported a value for the albumin trail of about 1 μg. per sq. cm. with Whatman 3 MM paper (uncorrected for paper blank). These investigators, however, employed bovine albumin in their studies. It was found by the present authors that crystalline bovine albumin (Armour) left a trail about 25 per cent less than human albumin.

Summary

Albumin is underestimated because of loss by adsorption onto the filter paper as the component migrates. Since the amount adsorbed appears to be constant over a considerable range of albumin concentration, the relative magnitude of underestimation increases as the albumin concen-

tration decreases. Furthermore, the globulins are overestimated because they are superimposed on the albumin "trail."

The degree of "trailing" was found to vary with the paper used.

Ionic Strength of the Buffer

For routine serum fractionations most workers employ veronal-sodium veronal buffer at *p*H 8.6 and with an ionic strength (μ) between 0.05 and 0.10. Fractionations obtained with three values of μ, namely 0.05, 0.083, and 0.10, were compared and no significant difference was found in the staining. Use of the 0.05 μ buffer yielded consistently poor resolution of components and frequently the α_1-globulin did not separate well from the albumin. Both of the higher buffer concentrations gave equally good resolutions. If polarity is reversed on alternate runs the buffer, left in the electrophoretic cell at room temperature, can be used for several months without alteration of results.

The ionic strength of the buffer with which the electrophoretic strips are wet was studied as to its effect on the subsequent staining of human albumin and human γ-globulin (Lot No. 2175-249-B, Poliomyelitis Immune Globulin, Lederle Lab. Division, American Cyanamid Co., New York City) and no significant difference observed over a range of μ from 0 to 0.166.

Summary

An ionic strength of 0.083 was adopted for the "standardized procedure" because of the good resolution obtained. Higher strengths required greater voltage or a longer time to achieve equal migration distances. The buffer, left in the electrophoretic cell at room temperature, can be used for several months if the polarity is reversed on alternate runs. Variations of ionic strength within the range tested appeared to have no effect on the quantitative results obtained except insofar as it affected resolution of components.

Sample Size and Protein Concentration

Samples were applied by use of the Spinco "striper," consisting essentially of two parallel wires which hold the sample by capillary action. A very even and narrow application is easily achieved. Nominally, the striper holds 10 μl. but actually it can be used to deliver from 5 to 15 μl. To determine the effect of variation of sample size, results with several normal sera using 5, 10, and 15 μl. samples were compared. With some sera there was no significant difference in results obtained with the 5 and

10 μl. sample sizes. With other sera, the A/G ratio obtained with 10 μl. appeared to be somewhat lower than that obtained with 5 μl. In all instances, however, the A/G ratio obtained with a 15 μl. sample size was lower (as much as 13 per cent) than that obtained with 10 μl. The implication that this observation has for reproducibility of results is clear and it is, therefore, essential that an exactly measured amount of sample be applied in striping.

As discussed previously the predicted effect of sample size on the error introduced by albumin "trailing" is the reverse of the observation just made. The postulate, therefore, was tendered that the dye uptake by protein was not strictly proportional to the amount of protein on the paper. Jencks, Jetton, and Durrum (2), employing essentially the same staining technic as used here, showed that a linear relationship exists between dye uptake and the quantity of protein up to 300 μg. An albumin concentration of 5 Gm./100 ml. is not infrequent in a normal serum and 10 and 15 μl. samples of such a serum would contain 500 and 750 μg. albumin, respectively. It appeared advisable, therefore, to investigate quantities greater than those studied by Jencks *et al.*

Twenty-microliter stripes of various dilutions of a normal serum were applied to filter paper wet with buffer, stained, and quantitated by elution. This volume was used rather than 10 μl. because the resultant protein area more closely approximated that occupied by the albumin component of an electrophoretic pattern. It was found that albumin quantities of 350, 400, 500, 600, 700, and 800 μg. were under estimated by 0, 4, 11, 17, 24, and 31 per cent, respectively. Such values cannot be used as a basis for corrections without considerable difficulty for, obviously, the protein concentration per unit area or mass of paper is the critical variable, a view in agreement with Guillien and Herman (6). The experiment does demonstrate, however, that the observed effect of variation of sample size can be accounted for by deviation of dye uptake from linearity when the protein concentration exceeds a certain limit. Such a deviation has been reported by others employing different staining technics (5, 6, 7, 8). With the staining technic employed herein it would appear that the only component of a normal serum which may be underestimated from this cause is albumin.

Summary

Deviation from linearity of dye uptake by proteins was observed when the protein concentration per unit mass of paper exceeded a certain limit. Albumin appears to be the only component of normal sera which may be

underestimated from this cause when a 10 μl. sample of serum is applied as a stripe of the dimensions used in this study. Quantitative results may thus be affected by variation in the size of sample applied.

Time and Voltage

Two periods of time for an electrophoretic run were compared. The first was 7 hours which permitted sufficient time in a 8-hour working day to heat-fix the strips and put them in the dye for overnight staining. A total of 15 ma current was used for the 8 strips accommodated by the Spinco cell (8 strips should always be used whether samples are applied to all or not), requiring the application of about 144 v. In 7 hours the front of the albumin component has migrated about 6.5 cm. from the origin. The application of a higher voltage in an attempt to shorten the time required or to increase the migration distance actually had the reverse effect, because more heat is produced which results in an increase in the ionic strength of the buffer in the paper strips.

The second period of time was overnight, about 16 hours. With the application of 80 v. the current was about 9 ma for 8 strips and the albumin front migrated about 9 cm. from the origin. This greater migration distance provided a somewhat better separation of components.

No significant quantitative differences in results by these two technics were observed. The predictable differences due to variation in the albumin trail resulting from the two different distances of migration are within the experimental error of the method.

Summary

Electrophoretic runs in the Spinco cell gave equivalent results when carried out for 7 hours and overnight, although the latter was generally preferable because of better separation of components.

VARIABLES IN STAINING

Heat-Fixation of the Proteins

At the end of the electrophoretic run it is necessary to "fix" the proteins to the cellulose matrix so that that they will not be eluted during the staining procedure. In the technic adopted this is accomplished by heat. The effect of the time and temperature of this process on dye uptake was studied, using a forced-draft oven. Table 1 gives the results (average of duplicates) of a typical experiment in which replicate strips were heat-fixed at different temperatures for 30 minutes. In this instance, as in cer-

Table 1. EFFECT OF TEMPERATURE OF "HEAT-FIXATION" ON BINDING OF DYE

	Absorbance (A) of eluted dye, strips "heat-fixed" for 30 mins.			
	90°	*100°*	*120°*	*140°*
Albumin	0.671	0.805	0.900	0.930
α_1-globulin	0.059	0.071	0.042	0.037
α_2-globulin	0.210	0.208	0.179	0.151
β-globulin	0.189	0.180	0.160	0.120
γ-globulin	0.205	0.190	0.182	0.144
Total A	1.334	1.455	1.463	1.382
A/G ratio	1.01	1.23	1.59	2.03

tain others, the total dye uptake (Total A) did not appear to change significantly, the decrease in uptake by all the globulins with increasing temperature being about equal to the increased uptake by albumin. With other sera, however, the dye uptake by the globulins remained practically unchanged, resulting in an increased total dye uptake with increasing temperature. From the slope of the curve relating A/G ratio to temperature of heat-fixation in the range of 100 to 120° at an A/G ratio of about 1.5, it was calculated that a 1° increase in temperature produces approximately a 1% increase in the A/G ratio. Obviously, careful control of temperature in the heat-fixation step is necessary and requires the use of an oven which will hold within at least ±3° of the set temperature. Furthermore, the use of the forced-draft type of oven with its more uniform temperature seems advisable.

Variation in the heating of strips at 110° from 15 minutes to 2 hours resulted in an increase in A/G ratio as the heating time was increased. From curves relating A/G ratio to time of heating it was calculated that at about 110°, variation in heating time from 27 to 33 minutes would affect a normal A/G ratio by no greater than 1%. This increase in A/G ratio with increased time of heating was previously reported by Jencks, Jetton, and Durrum (2).

Summary

The amount of dye combined with albumin increases with increasing temperature and time of heat-fixation. The amount of dye combined with the various globulins, on the other hand, either remained practically constant or decreased with increasing temperature and time of heat-fixation. The time and temperature of heat-fixation are, therefore, critical variables in the quantitative results obtained and their close control is essential for reproducible results.

Different Lots of Dye

For a staining technic to be valid it is necessary that results be independent of the batch or source of the dye used. A failure in this respect has been reported for the dye Oil Red O in the staining of lipoproteins (9). Bromphenol blue has been shown by chromatography to contain trace impurities (2). Single lots from Harleco (lot 59, Harshaw Scientific Division of Harshaw-Chemical Co.) and Coleman and Bell (lot B 116, 471217, The Matheson Co. Inc., East Rutherford, N. J.) and three lots (Nos. 15326, 14046, and 11837) from National Aniline Division, Allied Chemical and Dye Corp., New York, were compared by staining replicate electrophoretic patterns of one normal and one abnormal serum and no significant differences in staining properties were observed.

Summary

Five lots of bromphenol blue from three sources were tested and no significant differences in results were observed.

Time of Staining

Replicate electrophoretic patterns of several normal and abnormal sera were dyed for periods of 6 to 40 hours. Although the total dye uptake increased with the time of staining, the relative dye uptake for all protein fractions remained constant. This confirms the findings of Jencks *et al.* (2).

Summary

The relative dye uptake for all protein fractions was constant for staining periods from 6 to 40 hours.

Temperature of Staining

Staining at "room temperature" must include consideration of a temperature range from about 18° to 37°, the extremes of ambient temperature likely to occur. Replicate electrophoretic patterns of 3 normal and 2 abnormal sera were stained in duplicate at 18, 25, and 37°. Although the dye uptake decreased with increasing temperature, being as much as 15 per cent less at 37° than at 18°, the relative uptake by the various protein components remained constant.

Summary

The temperature of staining over the entire range of "room temperature" was found to be not critical.

Age of Dye Solution

The possible effects of age of dye solution and the number of strips previously stained therein were studied. Starting with a fresh batch of dye solution, replicate strips were stained at intervals between which the dye was employed for staining of routine samples. Results are given in Table 2. The dye solution (initial volume 1.2 L.) was kept continuously at room temperature (approximately 25°) in a polystyrene tray covered with a lid. Between February 12 and March 12 the volume of this solution had decreased to a low level and an equal volume of the original solution (unused) was added. Unused stain from this bottle which was stored at room temperature and in the dark was also tested on two occasions.

Study of Table 2 reveals the following points: (1) although the concentration of bromphenol blue (BPB) dropped from 10 to 3.3 mg. per 100 ml. after more than 122 strips had been stained and the total dye bound by the strips (A_{total}) decreased about 50 per cent, the relative dye uptake by the various protein fractions remained constant within experimental error; (2) the dye solution decomposes slowly at room temperature even in a stoppered bottle as shown by the drop in BPB concentration in the unused stain.

Summary

Within experimental error the relative dye uptake by the protein fractions was constant for the range of dye concentrations studied (3.3–10 mg. per 100 ml.). At least 100 strips can be stained with a 1.2 L. batch of dye solution. The authors discard a dye solution when the dye uptake of

Table 2. Effect of Age of Dye Solution on the Staining of Replicate Paper Electrophoretic Strips

	Date	*Jan. 16*	*Jan. 20*	*Jan. 26*	*Feb. 12*	*Mar. 12*	*Unused stain*	
							Jan. 26	*Mar. 12*
	Strip no.	1	33	66	122	226	1	1
	BPB conc. (mg. per 100 ml.)	10.0	7.7	5.5	4.0	3.3	7.8	7.2
% of total protein	Albumin	57.8	58.2	61.4	57.9	60.1	62.1	61.0
	α_1-glob.	4.5	4.7	3.1	4.3	3.2	3.2	2.8
	α_2-glob.	11.4	11.4	9.9	11.4	10.3	9.7	10.2
	β-glob.	10.9	10.6	11.1	11.8	10.4	10.4	10.8
	γ-glob.	15.4	15.1	14.5	14.6	16.0	14.6	15.2
	A/G ratio	1.4	1.4	1.6	1.4	1.5	1.6	1.6
	A_{total}	2.158	2.115	1.584	1.362	0.996	1.694	1.566

routine strips (A_{total}) has fallen to about one half of that obtained with a fresh dye solution.

Method of Washing Stained Strips

The washing technic outlined in the "standardized procedure" (steps 7 and 8) was compared with that given by Jencks *et al.* (2). The latter procedure is as follows: wash in three changes of 2% (v/v) aqueous acetic acid for 5, 5, and 10 minutes, respectively, then "fix" for two minutes in 10% (v/v) aqueous acetic acid containing 2% sodium acetate trihydrate. Replicate strips from 6 normal and 6 abnormal sera were washed by these two technics and no significant difference was observed in the results obtained. Nor did there appear to be any difference in the amount of dye remaining in paper blanks (paper devoid of protein).

The method employed in the "standardized procedure" gave stained strips of a purer blue color following heat drying than the alternate method which yielded strips of a blue-green color. For quantitation by elution this is of no consequence but for quantitation by photometric scanning the blue-green color must be converted to the blue by passing the strip over an opened bottle of concentrated ammonium hydroxide.

Summary

Two technics of washing stained strips were compared and no significant difference in results was observed except the color of the dye upon drying by heat.

Drying of Strips following Washing and Fixing

Following the washing and fixing procedure the strips are ordinarily heat-dried. The heat volatilizes the acetic acid, leaving a residue of the nonvolatile sodium acetate which, being alkaline in reaction, brings out the blue color of the dye. Drying at 107° for periods of 15 minutes to 3 hours resulted in no alteration of results. Actually, the strips can be dried at room temperature and the blue color brought out with ammonia fumes.

Summary

The time and temperature of drying following staining are not critical.

QUANTITATION OF STAINED ELECTROPHORETIC STRIPS

Quantitation of stained electrophoretic strips can be done either by photometric scanning (transmission or reflectance measurements) or by

cutting out the fractions, one segment for each fraction, eluting the dye, and quantitating the dye by photometry.

If the dye in the paper obeys Beer's law and if the amount of dye present is directly proportional to the amount of protein present, then the area under the densitometer curve is directly proportional to the amount of protein present. It has already been seen that the second premise is invalid above a certain limiting concentration of protein in the paper with the staining technic used. Jencks *et al.* (2) found that the dye, bromphenol blue, applied to the filter paper does not obey Beer's law. This was confirmed by the present authors employing a Beckman Model B spectrophotometer equipped with the Beckman scanning attachment. Dilutions of bromphenol blue in aqueous 1% sodium acetate applied as stripes to a filter paper (Whatman 3 MM) strip and scanned at 520, 540, and 600 mμ, either with the strip dry or oiled with mineral oil, showed considerable deviation from linearity when area under the scanning curve was plotted against dye concentration. Scanning of the unoiled strips was performed with half band-widths of about 18 mμ, whereas the oiled strips passed sufficiently more radiant energy to permit use of a half band-width of about 5 mμ. From the experiments with bromphenol blue alone it would be predicted that the area under the densitometer curve of stained protein would increase at a slowing rate as the protein concentration increases. This was confirmed by experiments in which twofold dilutions of sera were applied as stripes to filter paper, stained, and scanned.

Deviation from Beer's law by photometric scanning has been demonstrated by many groups of workers (e.g., 2, 8, 10) and, in fact, due to the heterogeneity of the paper matrix, deviation would be predicted (2, 11). As yet unexplained, however, are some claims (12, 13, 14) of finding an obedience to Beer's law.

As previously reported (15), oiling of electrophoretic strips had a very significant effect on the results obtained. For example, in the case of a pattern of an abnormal serum, the A/G ratio of the oiled strip was about 60 per cent higher than that obtained on the unoiled strip whether scanned at 540 or 600 mμ (half band-width 5 mμ).

Due to the deviation from linearity between the amount of a protein component present in an electrophoretic pattern and the corresponding area under the densitometer curve, the curve must be corrected before areas are determined if the relative proportions of the various protein components are to approximate those obtained by Tiselius electrophoresis or salt fractionation. This can be done manually (16) or automatically by a scanner such as the Analytrol (Spinco).

In quantitation by the elution technic as outlined in the "standardized procedure," reading of the absorbance of eluates at 540 mμ was suggested. Peak absorbance of bromphenol blue in alkaline solution is at 595 mμ (7). The color of the eluates at this wavelength is too dense unless rather large volumes of eluting fluid are employed. With about a 6-ml. volume, reading at a wavelength of 540 mμ is satisfactory. If the absorbance (A) is greater than 0.7 (usually the case for a normal albumin concentration), a suitable dilution should be made. On the other hand, in the case of relatively light staining resulting from short staining periods or the use of an old dye solution, absorbance readings can be brought into the optimal range by reading at a longer wavelength, e.g., 560 mμ. Measurements made with two spectrophotometers, a Bausch and Lomb Spectronic 20 and a Beckman Model B (half band-widths of 20 mμ or less), obeyed Beer's law. Significant deviations from Beer's law, however, were observed when using filters Nos. 54 and 60 (Corning Glass filters) with a Klett-Summerson photometer. Routine use of such a filter photometer, therefore, would require correction of apparent dye concentrations by a calibration curve. The dye faded appreciably within one hour in 0.1N or 0.03N NaOH but its relative stability in 0.01N NaOH (2) was confirmed. In this concentration of alkali the fading was no greater than 2% within one hour.

To determine the comparative reproducibilities of the scanning and elution technics, 16 replicate strips were run simultaneously and divided into two equal groups by random selection. All strips were stained and washed simultaneously. The strips in the group for elution were marked individually for cutting. Scanning of the second group was carried out by Dr. John Mehl, University of Southern California, using an Analytrol

Table 3. Comparison of the Elution and Scanning Technics

Serum fraction	*% of total protein ($\bar{x}$)*		*Reproducibility, 95% confidence limits in ± % of fraction*				
						Significant at	
	By elution	*By scanning (Analytrol)*	*Elution technic*	*Scanning technic*	*F*	*5% level*	*1% level*
Albumin	45.3	47.9	6.1	[a]	..	..	..
α_1-globulin	4.7	4.3	12.8	25.7	3.85	Yes	No
α_2-globulin	11.0	10.6	6.0	22.7	14.2	Yes	Yes
β-globulin	14.8	13.1	6.8	21.2	9.75	Yes	Yes
γ-globulin	24.2	24.1	10.9	14.2	1.7	No	No
A/G ratio	0.83	0.92	10.4	[a]	..	..	..

[a] The strips were stained too deeply for precise measurement of the albumin peaks by the Analytrol. Since this results in an unfair estimate of the reproducibility of measurement of this fraction and the A/G ratio by scanning, these estimates are deleted.

scanner and marking off the densitometer curves by visual inspection of the strips. The results are shown in Table 3. The reproducibilities of the two technics were compared statistically by the F test (17) and the last two columns of Table 3 indicate in which instances the precision of the elution technic significantly exceeded that of the scanning technic. The confidence limits obtained for the scanning technic approximate those previously reported for the same instrument (18). The limits for the elution technic shown in Table 3 approximate the averages obtained in four similar experiments.

The mean values for the fractions obtained by the two technics were compared by the statistical method of Aspin and Welch (19) with the result that the values for both α_1- and β-globulin differed significantly at the 5% and 1% levels, respectively. Values for α_2- and γ-globulin agreed within experimental error.

Summary

For results obtained by a scanning technic to agree with results obtained by the elution technic it is necessary to correct densitometer readings for deviation from Beer's law. Comparison of results obtained by the elution technic with those obtained by the Analytrol scanner showed that the two technics yielded quite similar values but the elution technic possessed significantly greater precision.

STABILITY OF SERA

Sera were studied for stability at room temperature (about 25°) and refrigerator temperature (4°), the groups comprised of 13 and 26 samples, respectively. Both normal and abnormal protein compositions were represented.

Room temperature studies were continued for periods up to nine days with the following results: (1) with but one exception no changes in electrophoretic patterns were observed during the first three days although in many instances the sera developed considerable turbidity; (2) between three and nine days the specimens became very turbid and putrid, and changes developed in the paper electrophoretic pattern, including a well-defined stripe of denatured protein remaining at the origin, almost invariable disappearance of α_1-globulin, decreased mobilities of α_2- and β-globulins, and occasionally the appearance of new components. The denatured protein, which in nine days was as much as 25 per cent of the total protein, appeared almost invariably to be derived from the globulins since the A/G ratio remained constant if the denatured component was

Table 4. NORMAL VALUES OF SERUM PROTEIN FRACTIONS AS OBTAINED BY THE PAPER ELECTROPHORETIC TECHNIC

	Total Protein (Gm./100 ml.)	*Albumin*		*α₁-globulin*		*α₂-globulin*		*β-globulin*		*γ-globulin*		*A/G ratio*
		Gm./100 ml.	*% of total*	Gm./100 ml.	*% of total*	Gm./100 ml.	*% of total*	Gm./100 ml.	*% of total*	Gm./100 ml.	*% of total*	
$\bar{X}$	7.4	4.5	59.8	0.30	4.1	0.70	9.4	0.85	11.6	1.13	15.3	1.55
95% limits	6.3–8.5	3.5–5.5	52–68	0.20–0.40	2.4–5.3	0.51–0.93	6.6–13.5	0.64–1.06	8.5–14.5	0.74–1.70	10.7–21.0	1.1–2.2

included with the globulins. The marked changes in α- and β-globulins observed by the classical Tiselius technic (20) were not seen by paper electrophoresis.

Studies at refrigerator temperature were carried out for periods up to four months with the following results: (1) no changes were observed during the first month although in some instances turbidity developed; (2) although some sera evidenced no change even after four months, usually some changes developed sometime after the first month. These changes were quite variable and included the development of denatured protein, the disappearance of α_1-globulin, increase of α_1-globulin, increase in the A/G ratio, and the appearance of new components.

NORMAL VALUES

Determinations of total protein by a biuret method and protein fractions by the "standard" paper electrophoretic method were performed on 12 normal adult women and 12 normal adult men, most of whom were from the laboratory staff. Values of the mean ($\bar{x}$) and 95% limits determined by the "normal equivalent deviate" method (21) are given in Table 4.

DISCUSSION

It has been demonstrated that the various serum protein fractions do not bind dye equally on a weight basis (2). Furthermore, it has been claimed that the dye-binding of a fraction can vary considerably in abnormal sera (22). The use of factors to correct for this variability would appear to be questionable and rather impractical.

Undeniably, there would be justification for correction of results for albumin trailing and this has been suggested (5). Calculations from the experimental data obtained indicate that such a correction would increase A/G ratios by about 12 to 20 per cent. It is the opinion of the authors, however, that this would unnecessarily complicate the procedure

and such a correction, therefore, was omitted from the routine procedure adopted.

On the theory that any charged groups on the paper which will bind dye will also bind protein and become inaccessible to dye, it has been suggested (2) that a paper blank should not be subtracted from each protein fraction. In this study, however, such a blank correction was made routinely. Omission of this correction would lower A/G ratios by about 5 to 10 per cent.

As can be seen from the results obtained in this study, selection of different procedural conditions will produce different normal ranges for the various protein fractions. To a great extent the choice of a particular set of conditions would appear to be quite arbitrary. The conditions which were adopted for a routine procedure represent a rather remarkably fortuitous balancing of the many variables involved to produce results such that the normal ranges obtained closely approach those reported for the classical Tiselius technic (23).

In the authors' opinion, the staining technic employing the dye solution of Jencks *et al.* (2) possesses certain advantages over the use of an ethanolic dye solution saturated with mercuric chloride as employed by many authors: the danger of exposure to mercury and the problem of its disposal are obviated; use of the ethanolic dye solution is materially more expensive because of the large amounts of mercury salt required and the relatively large amounts of ethanol used in the wash procedure; the dye is more easily eluted from protein on the strips.

SUMMARY

Investigation of various steps involved in separation of serum proteins by paper electrophoresis and their quantitation by dyeing with bromphenol blue has revealed that the results obtained are quite dependent on a number of variables. It is essential, therefore, that rigid control of these variables be exercised and that normal values be obtained by the specific technic adopted.

Normal values have been given for the technic employed and data on stability of sera at room and refrigerator temperatures have been presented.

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the Clinical Chemist

EDITORIAL

Two seemingly unrelated events have occurred within the past year. One of these is the International Congress of Clinical Chemistry held recently in New York City. The other is the publication of *Medical Research: A Midcentury Survey*, reviewed in another section of this issue.

The scientific sessions of the International Congress speak eloquently for the position this specialty has achieved in the advancement of chemical and medical research. Papers were presented by scientists from seventeen different countries and from four continents. The observation that many of the pioneers in this field are still actively engaged in their pursuits is evidence of the youthful vitality of clinical chemistry.

It is futile to speculate as to what medical research at midcentury might have been without the contributions of biochemistry and clinical chemistry. It would be more rewarding, however, to evaluate the importance of clinical chemistry in the present and future status of medical research.

The second volume of *Medical Research: A Midcentury Survey* deals with a group of nine yet unsolved clinical problems. The group could have readily been extended to include various other diseases. The chemist cannot, of course, attempt the solution of any of these within his own discipline. But it is realistic to assume that he will contribute to the elucidation of many of these disease processes.

This comprehensive work, published by the American Foundation Studies, would be valuable reading for everyone interested in the basic medical sciences. The clinical chemist will feel very much at home throughout the complete text. His contributions to medical research at midcentury have been duly noted.

MAX M. FRIEDMAN

REPORTS FROM THE SECTIONS

New York

First Van Slyke Award and Lecture

The first Van Slyke Award in Clinical Chemistry is to be presented to Dr. Donald D. Van Slyke, of Brookhaven National Laboratories, by the Metropolitan-New York Section of the AACC on Tuesday evening, February 26, at 8:30 p.m. at the Blumenthal Auditorium of the Mount Sinai Hospital, 100th Street and Fifth Avenue, New York City. The presentation will be made by Dr. Harry Goldenberg, Chairman of the section.

Dr. Van Slyke will deliver the first lecture of this new award series, "Some Phases of Oxygen Physiology."

At the last National Meeting of the AACC the New York Section received permission from the National Executive Committee to establish an award in clinical chemistry. Dr. Van Slyke, the section's most noted member, consented to have the award in his name and to be its first recipient. The occasion is to be another milestone in the history of the profession of clinical chemistry, and the New York Section cordially invites all members of the Association and fellow scientists to attend.

Ernst Bischoff Award Nominations

Members of the American Association of Clinical Chemists are again urged to submit nominations for the 1957 Ernst Bischoff Award. The deadline for such nominations is March 31, 1957.

Five copies of a résumé are to be submitted to the National Secretary, Dr. Max M. Friedman, Lebanon Hospital, New York 57, N. Y. The summary should include an adequate statement in support of the recommendation, although a complete bibliography is not required.

The Award consists of a scroll, a medal, and an honorarium of $500. It is presented annually to "a chemist on the staff of a hospital or a clinical chemistry laboratory who has distinguished himself by achievement and devotion and has helped solve those chemical problems which arise daily in the practice of the medical arts."

The recipient will be selected from the nominations by a group of five individuals appointed by the Executive Committee of the Association.

Midwest

The following officers were elected at the June, 1956, meeting of the Midwest Section, to assume their respective offices on July 1, 1956:

Chairman: Kurt M. Dubowski, Iowa Methodist Hospital, Des Moines, Iowa

Vice-Chairman: George M. Maruyama, Medical Associates, Dubuque, Iowa

Secretary-Treasurer: Robert S. Melville, Veterans Administration Hospital, Iowa City, Iowa

Southern California

C. A. Dubbs

Election of officers was held at the final dinner meeting in Los Angeles on June 5. Kenneth D. Johnson of the Chaney Chemical Laboratory succeeded Rex Sterling as chairman. William R. Bergren, University of

Southern California Medical School and Childrens Hospital, was elected program chairman and chairman-elect. Charles Sobel, Bio-Science Laboratories, was elected secretary-treasurer. Arnold Ware, University of Southern California Medical School and Los Angeles County Hospital, was elected chairman of the membership committee, with Otto Lobstein, Chem-Tech Laboratories, and Frank Martuccio, Martuccio Medical Laboratory, as members.

Merle L. Lewis, chairman of the Legislative Committee, reported that the California Department of Public Health, which administers the California Clinical Laboratory Act, expresses no objection to the principle of granting special licenses at the technologist (supervisory) level. This would permit qualified specialists such as clinical chemists to supervise a laboratory performing analytical work in its specialty field alone. However, the California Attorney General had earlier given an opinion [CLINICAL CHEMISTRY **1**, 134 (1955)] that the present law, which contains some indecisive and contradictory provisions, does not properly permit such licensure. The Committee proposes a clarification of the law by a few word changes to make such special licensure possible.

Cleveland

The following officers for the coming year were elected at the May meeting of the Cleveland Section:

Chairman: J. Waide Price, Institute of Pathology, Cleveland, Ohio

Vice-Chairman: Adrian Hainline, Jr., Cleveland Clinic, Cleveland, Ohio

Secretary: Irving Sunshine, Western Reserve University, Institute of Pathology, Cleveland, Ohio

Philadelphia

Peace Paubionsky

The 40th Scientific Session of the Philadelphia Section was held at the Physical Sciences Buildings of the University of Pennsylvania on October 30, 1956. Professor N. F. Maclagan of the University of London presented a talk on "Flocculation Tests in the Study of Hepatic Diseases." The discussion dealt with the use of turbidity and flocculation tests in differential diagnosis of hepatic diseases, the value of pairing test results in diagnosis, and the appearance and disappearance of positive findings as related to the clinical course of the disease. He also discussed a turbidity-flocculation modification of the original Takata-Ara test which had been developed by his co-workers. The test is more sensitive than the thymol turbidity and may be used as a screening test.

Professor Maclagan is well known for his work in the field of liver physiology and its clinical applications. More recently he has been investigating urinary mucoproteins.

The 41st Scientific Session was held also at the University of Pennsylvania on November 27, 1956, and was preceded by a brief report by John G. Reinhold on the successful International Congress of Clinical Chemistry held in New York, September 10–14.

The determination of serum trans-

aminase was discussed by Dr. Joseph H. Boutwell of the National Naval Medical School in Bethesda. Various aspects of the spectrophotometric technic were discussed, such as linear response, instability of reagents, and the effects of temperature and *p*H.

An alternative colorimetric procedure for the transaminase determination was presented by Dr. Wayne Umbreit of the Merck Institute for Therapeutic Research. Basic to the procedure is the possibility of differentiating a mixture of keto-acids reacting with 2,4-diphenylhydrazine. Some of the aspects discussed were spectral characteristics, preclusion of certain blank effects, and practical hints in extracting the color with organic solvents.

Interpretation of serum transaminase values was discussed briefly by Dr. David Seligson of the Graduate Hospital in Philadelphia. Emphasis was placed on the need for serial determinations, and certain sources of interference such as ingestion of meals and injection of bromsulfalein dyes.

Texas

The Texas section met at the Brooke Army Medical Center, Fort Sam Houston, on October 27, 1956. A symposium on the determination and significance of steroids was held under the chairmanship of Joseph W. Goldzieher. There were 168 registrants at the session. The presentations were well received and discusssions had to be curtailed because of lack of time.

At the business meeting Major José A. Rivera appointed Captain Dixon to the membership committee. A nominating committee will be appointed to bring a report at the December meeting.

The officers of the Texas section are:

Chairman: José A. Rivera, Brooke Army Medical Center

Chairman-Elect: Morton F. Mason, Parkland Hospital, Dallas

Secretary-Treasurer: Ferrin B. Moreland, Department of Biochemistry, Baylor University College of Medicine, Houston 25, Texas

Program Committee: Morton F. Mason, Chairman, Gerard F. Lanchantin, and Henry O. Nicholas

Membership Committee: Joseph H. Gast, Chairman, Arthur C. Dixon, and John K. Kirby

Washington-Baltimore-Richmond

The following are officers of the Washington-Baltimore-Richmond section for 1956–57:

Chairman: Elizabeth Frame, National Institutes of Health, Bethesda

Secretary-Treasurer: J. V. Princiotto, Georgetown University Medical School

Counselors: Miriam Reiner, Martin Rubin, and Ralph E. Peterson

Boston

At the October meeting of the Boston section the following officers were elected:

Chairman: William Cohen, Worcester Memorial Hospital

Vice-Chairman: Moira Reynolds, Boston University School of Medicine

Secretary-Treasurer: Marion K. Sherman, North East Baptist Hospital, Boston, Mass.

BOOK REVIEWS

Medical Research: A Midcentury Survey. Vol. I. *American Medical Research: In Principle and Practice,* 765 pp. Vol. II. *Unsolved Clinical Problems: In Biological Perspective,* 740 pp. Boston, Little, Brown and Company for the American Foundation, $15.00.

There is no medical science per se—it exists merely as applications of chemistry, physics, and mathematics in biology. It follows that medical research really is a product of research in these basic sciences. The prime importance of basic research in the advance of medicine is at once apparent. This concept became the guiding principle of the study undertaken by Esther Everett Lape and her staff at the American Foundation, with the aid of twenty-six distinguished consultant scientists. These volumes constitute the report of their comprehensive survey.

The first volume begins with a logical exposition of the relationship of biological, chemical, physical, and mathematical science to medical research. Emphasis is placed on expanding biological concepts in medicine and the interrelationships of the basic sciences in the evolution of these concepts. There follows an exhaustive review of the agencies that conduct research: universities, medical schools, foundations, and institutes. The auspices under which research is conducted are carefully analyzed. Finally, there is a report on how research is supported, with a thorough discussion of private and government means of financing as well as the influence of government.

The second volume consists of a searching review of nine major baffling subjects: cancer, infertility, arteriosclerosis, hypertension, the rheumatic syndromes, tuberculosis, the nature of viruses and of virus diseases, alcoholism, and biology of schizophrenia. Each problem is thoroughly explored, including background, current state, and anticipations of the future. These essays present facts succinctly and simply. They will enable an investigator to garner a comprehensive picture of the status in fields other than his own.

This work constitutes a rare blend of the gathering of information and the sympathetic understanding of related problems. The presentation of the case for basic research is both factual and logical. It must represent an articulation of the thoughts of many scientific investigators. Maurice Arthus said, "Seek facts and classify them—and you will be the workmen of science. Conceive or accept theories—and you will be their politicians." These volumes give the "workmen of science" a much-needed public hearing.

The wealth of information, the depth of comprehension, and the clarity of exposition are a solid tribute to the authors. This book is timely and necessary in these days of expanding private and governmental financial support to re-

search. It is highly recommended as essential reading to anyone concerned with medical research, be he student, administrator, educator, or investigator.

Lebanon Hospital, New York, N. Y. IRVING M. RATNER

Polarographic Techniques

L. MEITES. New York, Interscience Publishers, 1955, 317 pp., $6.00

This is an excellent text designed to give those interested in this relatively new technic an understanding of the theoretical fundamentals and, by the performance of the included experiments given as illustrations to the text, the practical laboratory experience.

Dr. Meites has organized his work, by a good discussion of the instrumentation and then the theory of polarography, to lead the reader gradually into the field. At the end of each phase of the discussion the author illustrates the points by giving detailed directions for the performance of experiments that emphasize the text. The illustrations of the polarograms are good, as well as the figures showing defective polarograms together with their causes and suggested remedies.

A 45-page appendix contains a comprehensive critical listing of data on half-wave potentials and diffusion current constants of inorganic substances published before December, 1954.

Though Dr. Meites does not go into the application of the technic to biologic material, his work should be one of the foundations for many chemists who use and wish to use polarography in their respective fields.

Polypeptides Which Stimulate Plain Muscle

J. H. GADDUM (Ed.) Baltimore, The Williams and Wilkins Co., 1955, 140 p., $3.75.

This book is based on a symposium organized by Professor U. S. von Euler which was held in Montreal, in 1953. It deals with a number of biologic substances obtained from tissues, which stimulate smooth muscle.

Fifteen papers are published, and the subjects range from investigations of kallekrein, a substance of animal origin which causes a fall in blood pressure and stimulates isolated smooth muscle, to cholecystokinin, a hormone released by the upper intestinal mucosa, which causes the gallbladder to contract and empty, and angiotonin (hypertensin), a pressor substance. Though the title of the book mentions only "polypeptides" a number of the substances discussed are not, but are included as they present similar chemical problems.

The authors present a clear discussion of the chemistry, physiology, and pharmacology of these tissue extract substances. Researchers interested in a new active compound or group can gain much by reading this work and profiting from the experiences of investigators reporting on similar types of investigations. Heretofore, this information has been scattered and is now brought together for the first time.

Metropolitan Hospital, New York, N.Y. H. D. APPLETON

ABSTRACTS

Editor: Ellenmae Viergiver. *Contributors:* Joseph S. Annino, Gladys J. Downey, Clyde A. Dubbs, Alex Kaplan, Margaret M. Kaser, Miriam Reiner, Herbert Thompson

Detection of prostatic acid phosphatase in serum by tartrate inactivation technic. E. H. Bensley, A. Drysdale, and R. Osiek (*Montreal General Hospital, Montreal, Canada*).

Prostatic acid phosphatase was determined by measuring acid phosphatase concentration before and after treatment with L-tartrate. The activity of the prostatic enzyme is reduced more than 90 per cent by such treatment while the activity of the acid phosphatase of normal serum and red cells is unaffected. A study of 375 patients revealed that inactivation of more than 1.0 unit/100 ml. lends support to a diagnosis of carcinoma of the prostate. However, in 94 tests on patients with prostatic carcinoma, 54 had tartrate-inactivated activity of 1.0 unit/100 ml. or less. Inactivation of a similar amount also occurs in a variety of nonprostatic diseases and is without diagnostic significance.—*Am. J. Clin. Pathol.* **26,** 247 (1956). (H. E. T.)

Evaluation of a rapid micromethod for the determination of serum calcium. H. C. Leifheit (*New England Medical Center, Boston, Mass.*).

A simple titrimetric method for the rapid estimation of calcium in 0.1 ml. of serum is based on the chelation powers of disodium ethylenediaminetetraacetate with Eriochrome Black T as the indicator. Since magnesium reacts in a similar fashion, the calculation from the titration of the serum gives the sum of the calcium and magnesium concentrations. The calcium concentration is then estimated by subtracting a mean value of 1.64 mEq./L. for magnesium. The assumption of this value is the chief weakness of the method.—*J. Lab. Clin. Med.* **47,** 623 (1956). (G. J. D.)

A microassay for plasma fibrinogen. H. D. Wycoff (*National Cancer Institute, University of Wisconsin, Madison, Wis.*).

To 0.1 ml. plasma in 15- x 85-mm. tubes add 2 ml. of thrombin solution (10 mg. Topical Thrombin in 100 ml. of 10% alcoholic saline) and mix quickly. The coagulation of fibrinogen is complete in 3 minutes and fibrin should be collected within an hour in glass "needles" 0.3 to 0.5 mm. x 3 in. Wash the collected fibrin by placing the needle in distilled water for 1 to 2 minutes, then in ethanol for 1 to 2 minutes. The amount of fibrinogen may then be determined by either a

gravimetric or a colorimetric procedure. Gravimetric method: dry the needle and fibrin at 80° for 30 minutes and weigh. Colorimetric method: place the needle in a colorimeter tube and digest the fibrin with 0.15 ml. of concentrated sulfuric acid in a sand bath for 5 minutes. Add 1 drop of 30% hydrogen peroxide with agitation and heat in the sand bath; repeat and allow tubes to remain in the sand bath for 5 to 10 minutes. Remove and cool. To each tube add 6.0 ml. of water, mix, and add 4.0 ml. of Nessler's reagent. Mix by shaking. Read at 500 mμ against a reagent blank and compare with a standard ammonium sulfate solution.—*J. Lab. Clin. Med.* **47,** 645 (1956). (G. J. D.)

Determination of total 17-hydroxycorticoids in plasma. W. J. Reddy, N. A. Haydar, J. C. Laidlaw, A. E. Renold, and G. W. Thorn (*Peter Bent Brigham Hospital, Boston, Mass.*).

The total 17-hydroxycorticoids in plasma are extracted with butanol and evaluated colorimetrically by the Porter-Silber reaction. The method is relatively simple and can be carried out in 1 day. A good correlation with known states of adrenocortical function has been demonstrated.—*J. Clin. Endocrinol. and Metabolism* **16,** 380 (1956). (M. R.)

Studies on obesity: I. The insulin-glucose tolerance curve. E. C. Arendt and C. J. Pattee (*Queen Mary Veteran Hospital, Montreal, Canada*).

In 32 obese patients given 0.1 unit of insulin per kg. intravenously followed in 30 minutes by 0.8 gm. of glucose per kg. orally the blood sugar level fell to 50 per cent of the initial value followed by a maximum rise to 164 per cent at 120 minutes. The level was still elevated after 180 minutes. In contrast, the mean response in 20 normal subjects consisted of a fall to 38 per cent followed by a maximum rise to 114 per cent at 90 minutes and a return to a value below the fasting level before 180 minutes. The differences in the curves were statistically significant.—*J. Clin. Endocrinol. and Metabolism* **16,** 367 (1956). (M. R.)

Studies in obesity: II. Blood pyruvate and lactate curves after ingestion of glucose. E. C. Arendt and C. J. Pattee (*Queen Mary Veteran Hospital, Montreal, Canada*).

The blood pyrivic acid and lactic acid levels after the ingestion of glucose by 21 obese and 10 normal subjects are reported. There was no significant difference between the pyruvate levels in the obese and in normal subjects. The rise in the lactate-pyruvate ratio in the obese group at the end of 3 hours was due to a significant increase in the blood lactic acid level.—*J. Clin. Endocrinol. and Metabolism* **16,** 375 (1956). (M. R.)

Interrelationships of glucose and inorganic phosphorus in blood and urine of patients with diabetes mellitus. J. L. Izzo (*University of Rochester, Rochester, N. Y.*).

The subjects were a group of 15 diabetic patients who varied in age and in the duration and severity of their diabetes. Throughout the experimental period they received a constant diet, and they were studied under optimal and suboptimal insulin regulation. Daily determinations were made of the fasting blood glucose and of the plasma or serum inorganic phosphorus in the same blood samples. Urinary glucose and phosphorus excretion were also studied. The range of blood glucose values was from 30 to 540 mg./100 ml., and the range of inorganic phosphorus was from 1.8 to 5.6 mg./100 ml. In all the patients a reciprocal relationship was found between the levels of fasting blood glucose and the inorganic phosphorus. However, the coefficient of correlation varied markedly from patient to patient. Glycosuria appeared to have no relationship to the phosphate changes, and only a very low positive correlation was found between urinary glucose and phosphorus. The findings concerning plasma glucose and phosphorus are the opposite of those usually reported for short-term experiments with glucose and/or insulin administration. It is believed that altered equilibria between phosphate stores and circulating phosphate are involved rather than any renal mechanism.—*Proc. Soc. Exptl. Biol. Med.* **91,** 373 (1956). (M. M. K.)

Prostatic serum acid phosphatase in patients with localized prostatic cancer. J. H. Hill (*University of Kansas Medical School, Kansas City, Kans.*).

Abnormally elevated total serum acid phosphatase is diagnostic of prostatic cancer with metastases in about 60 per cent of patients with this condition. This study was designed to determine whether or not patients having untreated carcinoma of the prostate without demonstrable metastasis could be identified by the degree of concentration of prostatic serum acid phosphatase. Although further improvements in the technic of measuring prostatic serum acid phosphatase may alter results, its superiority in the diagnosis of apparently localized prostatic cancer over total serum acid phosphatase has not been conclusively established.—*Am. J. Clin. Pathol.* **26,** 120 (1956). (H. E. T.)

Spectrophotometric determination of the oxygen saturation of whole blood. M. U. Tsao, S. S. Sethna, C. H. Sloan, and L. J. Wyngarden (*University of Michigan Medical School, Ann Arbor, Mich.*).

Blood is drawn in a heparinized syringe and 0.5 ml. is transferred anaerobically to a 2-ml. syringe. The sample is hemolyzed immediately with 0.01 ml. of a saponin solution and transferred to a special cuvet. The optical density is

determined with a Beckman DU spectrophotometer at 576 mμ, which represents the oxyhemoglobin concentration, and at 505 mμ, which represents the total hemoglobin concentration. The ratio of the reading at 576 mμ to that at 505 mμ is calculated and the oxygen saturation of the blood is read directly from a graph. The graph is constructed by plotting the ratio as the abscissa and the percentage saturation as the ordinate for known samples of blood. Good correlation was obtained with results using the Van Slyke manometric procedure.—*J. Biol. Chem.* **217,** 479 (1955). (J. S. A.)

Estimation of total free reducing plasma steroids. C. Chen, S. M. Voegtli, and S. Freeman (*Northwestern University Medical School, Chicago, Ill.*).

The steroids are extracted from 10 to 15 ml. of plasma with sodium chloride and methylene chloride solutions. The extracts are washed, dried, and evaporated to about 1 ml. volume. The concentrate is applied to a strip of specially treated chromatographic filter paper and is subjected to ascending chromatography in several controlled steps using methanol. The sample is eluted with a solution of absolute ethanol, blue tetrazolium, and choline, and the density is determined in a colorimeter at 515 to 520 mμ. Recovery data and normal values are given.—*J. Biol. Chem.* **217,** 709 (1955). (J. S. A.)

A new serum iodine component in patients with functional carcinoma of the thyroid. J. Robbins, J. E. Rall, and R. W. Rawson (*Memorial Center for Cancer and Allied Diseases, New York, N. Y.*).

The nature of the serum iodine has been examined in 23 patients with highly functional carcinoma of the thyroid, after 13 tracer doses and 37 therapeutic doses of I^{131}. Although these tumors seem to synthesize normal thyroglobulin and secrete thyroxine and triiodothyronine into the blood, more than half the patients studied had in addition an iodine component, compound X, different from the products of normal thyroid metabolism. Compound X is a large molecule which may not be homogeneous, is partly soluble in acid butanol, is immobile in various chromatographic butanol systems, and resembles serum albumin in several respects. It can be hydrolyzed by crude pancreatic enzymes, with the release of a high proportion of monoiodotyrosine in some instances, and thyroxine in another. Compound X appears to represent a functional abnormality in certain thyroid carcinomas. There has been no correlation between various clinical and laboratory data and abnormal serum iodine.—*J. Clin. Endocrinol. and Metabolism* **15,** 1315 (1955). (M. R.)

Triiodothyronine in the serum of patients treated with radioactive iodine. R. S. Benua, B. M. Dobyns, and A. Nimmer (*Western Reserve University School of Medicine, Cleveland, Ohio*).

Labeled triiodothyronine has been identified by a chromatographic fractionation procedure in the sera of 27 of 52 patients treated with radioactive iodine.

It was recovered from 22 patients with Graves' disease, from 1 patient with a toxic nodular goiter, from 2 patients with remnants of thyroid tissue after nearly complete thyroidectomy for cancer, and from 2 euthyroid patients with heart disease. Triiodothyronine was detected as early as 1 hour after the administration of I^{131}. The data suggest that triiodothyronine in the serum was not the result of radiation damage or of the peripheral deiodination of thyroxine, but was released from the thyroid gland into the circulation.—*J. Clin. Endocrinol. and Metabolism* **15,** 1367 (1955). (M. R.)

Effect of intravenous injection of fructose, with and without ACTH administration, on the level of blood glucose. R. F. Tagnon and S. Devreux (*University of Brussels, Belgium*).

In normal subjects the intravenous injection of fructose did not result in any elevation of the blood glucose level. However, the administration of ACTH prior to the injection of fructose resulted in a significant elevation of glucose. It is believed that this indicates the conversion of fructose into glucose in the body. In diabetic patients the injection of fructose resulted in an elevation of the blood glucose with and without ACTH. The importance of determining the blood glucose level in patients receiving fructose intravenously is emphasized.—*J. Clin. Endocrinol. and Metabolism* **15,** 1475 (1955). (M. R.)

Diagnostic accuracy of serum protein-bound iodine determination in thyroid disease. C. M. Blackburn and M. H. Power (*Mayo Clinic, Rochester, Minn.*).

The diagnostic accuracies of the protein-bound iodine (PBI) determination, the basal metabolic rate, and the uptake of radioiodine were compared in over 1000 patients. The serum PBI level proved to be the most valuable single test in the clinical evaluation of thyroid function, often providing decisive diagnostic information when the results of other tests were inconclusive. However, in a small proportion of patients with abnormal thyroid function who had values for PBI within the normal range, other tests of thyroid function were unequivocally abnormal. Therefore, in difficult problems of the disease, the combined use of all tests of thyroid function may be necessary for accurate diagnosis.—*J. Clin. Endocrinol. and Metabolism* **15,** 1379 (1955). (M. R.)

The effect of suspended solids in thromboplastin preparations upon prothrombin determinations. G. E. Phillips and J. G. Lenahan (*Warner-Chilcott Research Laboratory, Morris Plains, N. J.*).

There is a definite correlation between the prothrombin time and the concentration of suspended particles in thromboplastin suspensions. The effect is small in normal whole plasma but becomes more obvious, and often critical, when the amount of prothrombin is reduced as it is in dilute normal plasma or in whole plasma from patients treated with anticoagulants. The amount of suspended

material should be controlled by turbidimetric determination in order to obtain comparable thromboplastin preparations.—*J. Lab. Clin. Med.* **46,** 713 (1955). (G. J. D.)

A quantitative modification of the "tubeless" gastric analysis. P. B. Donovan and W. J. Tighe (*Rees-Stealy Clinic, San Diego, Calif.*).

A quantitative correlation has been demonstrated between the 2-hour urinary quinine excretion in the Diagnex test and the amount of HCl aspirated in the conventional gastric analysis. The volume and concentration of each aspirated specimen was converted to milliliters of 0.1 N HCl. When the mean values of aspirated acid were plotted against the mean values of urinary quinine excretion, a straight line relationship was evident, which is represented by the equation: $X = (Y - 82)/7.3$, where X = ml. 0.1 N HCl aspirated and Y = μgm. of quinine.

By applying this formula to urinary quinine values, one can estimate the equivalent acid secretion, information which extends the usefulness of the Diagnex test.—*J. Lab. Clin. Med.* **46,** 895 (1955). (G. J. D.)

Correction of erratic reagent blanks in determination of serum protein-bound iodine. D. Sanshuk and L. Aconsky (*Walter Reed Army Medical Center, Washington, D. C.*).

Two serious sources of error in the alkaline ashing procedure of Barker, Humphrey, and Sobel [*J. Clin. Invest.* **30,** 55 (1951)] are the use of Cl ion either as NaCl or HCl, and the iodine impurity present in NaCl even though it is chemically pure. When NaCl and HCl are eliminated, the rate of decolorization of the ceric ion is rapid enough to take reliable readings over a 12-minute period. The reagent blanks show very little or no change in optical density during this period.

To compensate for the elimination of HCl from the procedure it is necessary to increase the sulfuric acid concentration until it is equivalent to the total of the HCl and H_2SO_4 in the original method. In the authors' laboratory, the ashed sample is dissolved in 3.6 ml. of 7N H_2SO_4 plus 5.4 ml. of distilled water.—*J. Lab. Clin. Med.* **46,** 902 (1955). (G. J. D.)

Hydrometer for quick and accurate determination of specific gravity in urine. T. B. Magath (*Mayo Clinic, Rochester, Minn.*).

The hydrometer is designed on the principle of the Mohr balance and is accurate to 0.001. The apparatus operates with a minimum of attention and repair, and readings can be made as rapidly as the urine can be handled. This apparatus eliminates the difficulties in obtaining accurate measurements with the use of conventional urinometers.—*Am. J. Clin. Pathol.* **25,** 1217 (1955). (H. E. T.)